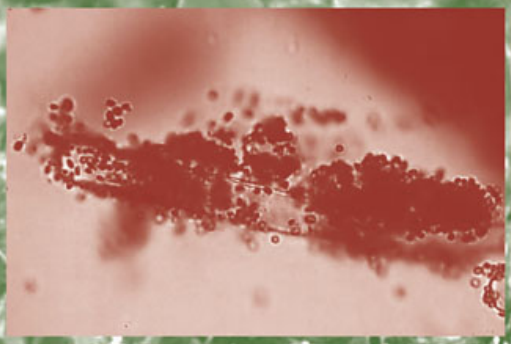
NITROGEN FIXATION: ORIGINS, APPLICATIONS, AND RESEARCH PROGRESS

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

Edited by
Claudine Elmerich and William E. Newton



Springer

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

Nitrogen Fixation: Origins, Applications, and Research Progress

VOLUME 5

The titles published in this series are listed at the end of this volume.

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

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PREFACE TO THE SERIES

Nitrogen Fixation: Origins, Applications, and Research Progress

Nitrogen fixation, along with photosynthesis as the energy supplier, is the basis of all life on Earth (and maybe elsewhere too!). Nitrogen fixation provides the basic component, fixed nitrogen as ammonia, of two major groups of macromolecules, namely nucleic acids and proteins. Fixed nitrogen is required for the N-containing heterocycles (or bases) that constitute the essential coding entities of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA), which are responsible for the high-fidelity storage and transfer of genetic information, respectively. It is also required for the amino-acid residues of the proteins, which are encoded by the DNA and that actually do the work in living cells. At the turn of the millennium, it seemed to me that now was as good a time as any (and maybe better than most) to look back, particularly over the last 100 years or so, and ponder just what had been achieved. What is the state of our knowledge of nitrogen fixation, both biological and abiological? How has this knowledge been used and what are its impacts on humanity?

In an attempt to answer these questions and to capture the essence of our current knowledge, I devised a seven-volume series, which was designed to cover all aspects of nitrogen-fixation research. I then approached my long-time contact at Kluwer Academic Publishers, Ad Plaizier, with the idea. I had worked with Ad for many years on the publication of the Proceedings of most of the International Congresses on Nitrogen Fixation. My personal belief is that congresses, symposia, and workshops must not be closed shops and that those of us unable to attend should have access to the material presented. My solution is to capture the material in print in the form of proceedings. So it was quite natural for me to turn to the printed word for this detailed review of nitrogen fixation. Ad's immediate affirmation of the project encouraged me to share my initial design with many of my current co-editors and, with their assistance, to develop the detailed contents of each of the seven volumes and to enlist prospective authors for each chapter.

There are many ways in which the subject matter could be divided. Our decision was to break it down as follows: nitrogenases, commercial processes, and relevant chemical models; genetics and regulation; genomes and genomics; associative, endophytic, and cyanobacterial systems; actinorhizal associations; leguminous symbioses; and agriculture, forestry, ecology, and the environment. I feel very fortunate to have been able to recruit some outstanding researchers as co-editors for this project. My co-editors were Mike Dilworth, Claudine Elmerich, John Gallon, Euan James, Werner Klipp, Bernd Masepohl, Rafael Palacios, Katharina Pawlowski, Ray Richards, Barry Smith, Janet Sprent, and Dietrich Werner. They worked very hard and ably and were most willing to keep the volumes moving along reasonably close to our initial timetable. All have been a pleasure to work with and I thank them all for their support and unflagging interest.

Nitrogen-fixation research and its application to agriculture have been ongoing for many centuries – from even before it was recognized as nitrogen fixation. The Romans developed the crop-rotation system over 2000 years ago for maintaining and improving soil fertility with nitrogen-fixing legumes as an integral component. Even though crop rotation and the use of legumes was practiced widely but intermittently since then, it wasn't until 1800 years later that insight came as to how legumes produced their beneficial effect. Now, we know that bacteria are harbored within nodules on the legumes' roots and that they are responsible for fixing N_2 and providing these plants with much of the fixed nitrogen required for healthy growth. Because some of the fixed nitrogen remains in the unharvested parts of the crop, its release to the soil by mineralization of the residue explains the follow-up beneficial impact of legumes. With this realization, and over the next 100 years or so, commercial inoculants, which ensured successful bacterial nodulation of legume crops, became available. Then, in the early 1900's, abiological sources of fixed nitrogen were developed, most notable of these was the Haber-Bosch process. Because fixed nitrogen is almost always the limiting nutrient in agriculture, the resulting massive increase in synthetic fixed-nitrogen available for fertilizer has enabled the enormous increase in food production over the second half of the 20^{th} century, particularly when coupled with the new "green revolution" crop varieties. Never before in human history has the global population enjoyed such a substantial supply of food.

Unfortunately, this bright shiny coin has a slightly tarnished side! The abundance of nitrogen fertilizer has removed the necessity to plant forage legumes and to return animal manures to fields to replenish their fertility. The result is a continuing loss of soil organic matter, which decreases the soil's tilth, its water-holding capacity, and its ability to support microbial populations. Nowadays, farms do not operate as self-contained recycling units for crop nutrients; fertilizers are trucked in and meat and food crops are trucked out. And if it's not recycled, how do we dispose of all of the animal waste, which is rich in fixed nitrogen, coming from feedlots, broiler houses, and pig farms? And what is the environmental impact of its disposal? This problem is compounded by inappropriate agricultural practice in many countries, where the plentiful supply of cheap commercial nitrogen fertilizer, plus farm subsidies, has encouraged high (and increasing) application rates. In these circumstances, only about half (at best) of the applied nitrogen reaches the crop plant for which it was intended; the rest leaches and "runs off" into streams, rivers, lakes, and finally into coastal waters. The resulting eutrophication can be detrimental to marine life. If it encroaches on drinking-water supplies, a human health hazard is possible. Furthermore, oxidation of urea and ammonium fertilizers to nitrate progressively acidifies the soil – a major problem in many agricultural areas of the world. A related problem is the emission of nitrogen oxides (NO_x) from the soil by the action of microorganisms on the applied fertilizer and, if fertilizer is surface broadcast, a large proportion may be volatilized and lost as ammonia. For urea in rice paddies, an extreme example, as much as 50% is volatilized and lost to the atmosphere. And what goes up must come down; in the case of fertilizer nitrogen, it returns to Earth in the rain, often acidic in nature. This

uncontrolled deposition has unpredictable environmental effects, especially in pristine environments like forests, and may also affect biodiversity.

Some of these problems may be overcome by more efficient use of the applied fertilizer nitrogen. A tried and tested approach (that should be used more often) is to ensure that a balanced supply of nutrients (and not simply applying more and more) is applied at the right time (maybe in several separate applications) and in the correct place (under the soil surface and not broadcast). An entirely different approach that could slow the loss of fertilizer nitrogen is through the use of nitrification inhibitors, which would slow the rate of conversion of the applied ammonia into nitrate, and so decrease its loss through leaching. A third approach to ameliorating the problems outlined above is through the expanded use of biological nitrogen fixation. It's not likely that we shall soon have plants, which are capable of fixing N_2 without associated microbes, available for agricultural use. But the discovery of N_2-fixing endophytes within the tissues of our major crops, like rice, maize, and sugarcane, and their obvious benefit to the crop, shows that real progress is being made. Moreover, with new techniques and experimental approaches, such as those provided by the advent of genomics, we have reasons to renew our belief that both bacteria and plants may be engineered to improve biological nitrogen fixation, possibly through developing new symbiotic systems involving the major cereal and tuber crops.

In the meantime, the major impact might be through agricultural sustainability involving the wider use of legumes, reintroduction of crop-rotation cycles, and incorporation of crop residues into the soil. But even these practices will have to be performed judiciously because, if legumes are used only as cover crops and are not used for grazing, their growth could impact the amount of cultivatable land available for food crops. Even so, the dietary preferences of developed countries (who eats beans when steak is available?) and current agricultural practices make it unlikely that the fixed-nitrogen input by rhizobia in agricultural soils will change much in the near-term future. A significant positive input could accrue, however, from matching rhizobial strains more judiciously with their host legumes and from introducing "new" legume species, particularly into currently marginal land. In the longer term, it may be possible to engineer crops in general, but cereals in particular, to use the applied fertilizer more efficiently. That would be a giant step the right direction. We shall have to wait and see what the ingenuity of mankind can do when "the chips are down" as they will be sometime in the future as food security becomes a priority for many nations. At the moment, there is no doubt that commercially synthesized fertilizer nitrogen will continue to provide the key component for the protein required by the next generation or two.

So, even as we continue the discussion about the benefits, drawbacks, and likely outcomes of each of these approaches, including our hopes and fears for the future, the time has arrived to close this effort to delineate what we know about nitrogen fixation and what we have achieved with that knowledge. It now remains for me to thank personally all the authors for their interest and commitment to this project. Their efforts, massaged gently by the editorial team, have produced an indispensable reference work. The content is my responsibility and I apologize

upfront for any omissions and oversights. Even so, I remain confident that these volumes will serve well the many scientists researching nitrogen fixation and related fields, students considering the nitrogen-fixation challenge, and administrators wanting to either become acquainted with or remain current in this field. I also acknowledge the many scientists who were not direct contributors to this series of books, but whose contributions to the field are documented in their pages. It would be remiss of me not to acknowledge also the patience and assistance of the several members of the Kluwer staff who have assisted me along the way. Since my initial dealings with Ad Plaizier, I have had the pleasure of working with Arno Flier, Jacco Flipsen, Frans van Dunne, and Claire van Heukelom; all of whom provided encouragement and good advice – and there were times when I needed both!

It took more years than I care to remember from the first planning discussions with Ad Plaizier to the completion of the first volumes in this series. Although the editorial team shared some fun times and a sense of achievement as volumes were completed, we also had our darker moments. Two members of our editorial team died during this period. Both Werner Klipp (1953-2002) and John Gallon (1944-2003) had been working on Volume II of the series, *Genetics and Regulation of Nitrogen-Fixing Bacteria*, and that volume is dedicated to their memory. Other major contributors to the field were also lost in this time period: Barbara Burgess, whose influence reached beyond the nitrogenase arena into the field of iron-sulfur cluster biochemistry; Johanna Döbereiner, who was the discoverer and acknowledged leader in nitrogen-fixing associations with grasses; Lu Jiaxi, whose "string bag" model of the FeMo-cofactor prosthetic group of Mo-nitrogenase might well describe its mode of action; Nikolai L'vov, who was involved with the early studies of molybdenum-containing cofactors; Dick Miller, whose work produced new insights into MgATP binding to nitrogenase; Richard Pau, who influenced our understanding of alternative nitrogenases and how molybdenum is taken up and transported; and Dieter Sellmann, who was a synthetic inorganic chemistry with a deep interest in how N_2 is activated on metal sites. I hope these volumes will in some way help both preserve their scientific contributions and reflect their enthusiasm for science. I remember them all fondly.

Only the reactions and interest of you, the reader, will determine if we have been successful in capturing the essence and excitement of the many sterling achievements and exciting discoveries in the research and application efforts of our predecessors and current colleagues over the past 150 years or so. I sincerely hope you enjoy reading these volumes as much as I've enjoyed producing them.

William E. Newton
Blacksburg, February 2004

PREFACE

Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations

This book is part of the seven-volume series that was launched a few years ago with the ambitious objectives of reviewing the field of nitrogen fixation from its earliest beginnings through the millennium change and of consolidating the relevant information - from fundamental to agricultural and environmental aspects – all in one place. Volume 5 covers the biology of bacteria that associate with non-leguminous plants. The subject matter includes a wide range of associations; it covers the bacterial species that associate either with the surface or within the tissues of grasses (often referred as plant growth-promoting rhizobacteria) and also the symbiotic associations that cyanobacteria form with fungi, algae, and both lower and higher plants. This volume does not deal with the *Frankia*-actinorhizal plant associations, which is the topic of Volume 6.

The book is divided in 13 chapters, each of which is the work of well-known scientists in the field. Just like in the other volumes of this series, the first chapter is an historical perspective. It describes how, as early as the end of the 19th century, it was shown that plant exudation favoured the proliferation of soil bacteria in the rhizosphere, and how the first nitrogen-fixing bacteria, including cyanobacteria were isolated. The chapter covers the landmarks and scientific concepts that arose from more than one century of research in this area.

Recently, implementation of the techniques of molecular phylogeny has led to the identification of an increasing number of N_2-fixing genera and species associated with grasses. The taxonomic status of both old and recently discovered species of the α- and β-subgroups of the Proteobacteria is the topic of the second chapter. Chapter 2 also outlines the ecology of these genera and then describes both tools and molecular probes that can be used for *in situ* localization of associated bacteria, in particular, to distinguish the bacteria located on the root surface from the endophytes resident within the plant tissues.

The genetics and regulation of nitrogen fixation in free-living bacteria is dissected in detail in Volume 2, however, it is of such importance that selected coverage of this subject is provided here in Volume 5, especially as it relates to the current understanding of the *nif* genetics of the most important grass-associated species; *Azospirillum brasilense*, *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus, Azoarcus sp.,* and *Pseudomonas stutzeri.* Indeed, Chapter 3 uses the established knowledge of *Klebsiella* and *Azotobacter nif* genetics as a basic framework on which to provide a comprehensive and comparative view of the grass-associated bacterial systems, while simultaneously emphasizing the unique features of each system and their regulatory networks.

Five chapters of Volume 5 focus on the molecular bases of the plant growth-promotion effect and the plant response to inoculation. Chapters 4 and 5 review more specifically the physiological and molecular bases of the root colonization. The molecular mechanisms of chemotaxis and the role of the chemotactic response

in adaptation to the soil and plant rhizosphere are reviewed in Chapter 4. Chapter 5 continues the colonization process, from attachment through to root-surface colonization, with a detailed review of the involvement of flagella, pili, and surface polysaccharide components. This chapter also presents a comprehensive analysis of the factors required for rhizosphere competence both at the physiological and genetic levels. Next, the idea that plants benefit from associated bacteria as a consequence of microbial phytohormone production was launched more than 50 years ago and this is the subject of Chapter 6. It is apparent that soil bacteria produce a wide range of plant hormones and that there is a multiplicity of biosynthetic pathways. For example, the routes for indole-3-acetic acid biosynthesis differ in plants, in pathogenic bacteria, and in plant-associated N_2-fixing bacteria. Chapter 6 describes this multiplicity of pathways and discusses the role(s) of these compounds in the association.

Chapter 7 reviews the overall plant response to inoculation, including the changes in root morphology, root metabolism, and effect on plant productivity. It also includes a review of the effect of *Azospirillum* and other bacterial inoculation on legume nodulation. To complete the presentation of plant-growth promotion by inoculation, Chapter 8 deals with the role of the N_2-fixing bacteria associated with grasses as biocontrol agents, even though the amount of information in the particular case of nitrogen fixers is still limited. Biocontrol is the property of beneficial bacteria to compete with pathogens through, for example, antibiosis, iron sequestration, or aggressive root colonization. The chapter also describes the mechanisms of activation of plant defences.

Although Chapters 4 to 8 include information on the colonization ability of a range of microorganisms, the main emphasis is on *Azospirillum* as the paradigm for root-surface colonization. With the discovery some 15 years ago of endophytic associations involving N_2-fixing bacteria that did not cause disease symptoms, a new research era arrived. The example of *Azoarcus* is treated in Chapter 9, which reviews the phylogeny and physiology of *Azoarcus* and related bacteria. It describes the cytology and the molecular biology of the interaction of *Azoarcus* with rice and Kallar grass. Chapter 10 deals with sugarcane-cropping systems and focuses on the diversity of N_2-fixing bacteria associated with sugarcane. It emphasizes the modes of endophytic colonization and the molecular biology of both *G. diazotrophicus* and *H. seropedicae*.

Cyanobacteria coverage is limited to two chapters, but additional information on the physiology, genetics, and genomics of cyanobacteria is given in Volume 2, *Genetics and Regulation of Nitrogen Fixation in Free-living Bacteria*, and Volume 3, *Genomes and Genomics of Nitrogen-fixing Organisms*. Because differentiation of the non-N_2-fixing vegetative cells into N_2-fixing heterocysts is crucial for a successful cyanobacterial symbiosis, Chapter 11 summarizes current knowledge of the physiology and genetics of filamentous cyanobacteria, emphasizing the differentiation process. This chapter is followed by a comprehensive and extensive review of the various plant associations involving filamentous cyanobacteria. Chapter 12 describes the biology of the different symbioses of cyanobacteria with diatoms, *Geosiphon*, lichens, liverworts, hornworts, mosses, *Azolla*, Cycads, and *Gunnera*. Volume 5 then concludes with a chapter describing the potential of

endophytic nitrogen fixers for the future and discusses the ideal model of a diazotrophic endophyte.

It took several years to compile the contents of this volume and to finalize the chapters. We give special recognition to all the authors, who shared their knowledge and ideas in this fascinating field, and we hope that their invaluable contributions will promote nitrogen-fixation and related research efforts and drive us onward to more spectacular discoveries in the future.

We give a special thought to Johanna Döbereiner, a leading figure in this field, who passed away in 2000. This volume is dedicated to her memory. Many researchers learnt from her and are proud to have done so; they continue to work in her spirit. We also remember Jean-Paul Aubert, deceased in 1997, for his support of nitrogen-fixation research for more than 20 years. Finally, we thank our families, friends, and colleagues for their interest and continual support during the time spent editing this volume.

Claudine Elmerich
Gif-sur-Yvette, April 2005

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Blacksburg, April 2005

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Johanna Döbereiner (1924 – 2000)

This volume is dedicated to the memory of Johanna Döbereiner in recognition of her forty-nine years of research in soil microbiology. Johanna Döbereiner was born in Czechoslovakia in 1924, she studied agronomy at the University of Munich and, in 1951, emigrated with her family to Brazil. She started work in the "soil microbiology laboratory" in the National Department for Agricultural Research of the Ministry of Agriculture in Seropédica, which later became the EMBRAPA. Johanna was at the centre of biological nitrogen-fixation research from the early discovery of *Azotobacter paspali* associated with the roots of *Paspalum notatum* until the "endophytic" associations of N_2-fixing bacteria within the tissues of forage grasses, cereals, and sugarcane. She published more than 500 scientific papers and she was ranked seventh among Brazilian scientists in the citations of her publications and the first amongst female scientists. But above all, those of us who understood her strong personality prized her friendship, her encouragement, and her capacity to face work as happy and enthusiastic as a person going on holiday. Johanna was more than a leader, she was a mother to many scientists (and a grandmother to the youngest), and she was a great friend and a source of pride for all of us. Johanna was awarded the degrees of Doctor *Honoris Causa* by both the University of Florida, USA, and the Universidade Federal Rural do Rio de Janeiro, plus the National Frederico de Menezes Viega Prize, the Bernard Houssay Prize, the UNESCO Science Prize, the Science and Technology Prize of Mexico, the Order of Rio Branco, the Order of Merit of the National Judiciary, and the Order of Merit of the Federal Republic of Germany. She was a member of the Academy of Sciences of the Vatican, the Brazilian Academy of Sciences, and the Third World Academies of Sciences. We thank V. Massena Reis, A. A. Franco, J. I. Baldani, M. C. Prata Neves, R. M. Boddey, V. L. Divan Baldani, and F. Pedrosa for supplying this dedication.

Chapter 1

HISTORICAL PERSPECTIVE: FROM BACTERIZATION TO ENDOPHYTES

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1. THE NITROGEN CYCLE: HERITAGE FROM THE 19TH CENTURY

The various steps of the nitrogen cycle and the major groups of microorganisms involved were discovered during the 19th Century (Figure 1; Table 1). Reyset, in 1856, was the first to describe the decomposition of organic matter in the soil that resulted in the release of nitrogen gas into the atmosphere, so providing the basis of the nitrogen cycle (see Payne, 1990). Schlossing and Müntz discovered the nitrification process in 1877 and Winogradsky obtained the first culture of *Nitrosomonas* by 1890. Gayon and Dupetit discovered denitrification in 1886 (Payne, 1990; Aubert, 1995). According to Wilson (1957), the notion of biological nitrogen fixation was born around 1837, although "gestation had been under way for many years". This idea, therefore, preceded the historical discovery of Hellriegel and Wilfarth, who established in 1886 that legumes, bearing root nodules induced by bacteria, could use gaseous nitrogen (Wilson, 1957; Nutman, 1987).

Even earlier, by 1771, Priestley was already convinced that plants could absorb nitrogen gas and this view was later adopted by many others (reviewed by Payne 1990). But scientists, including de Saussure and Liebig, challenged this view and declared that the fixed nitrogen originated only from the ammonia present in water, air, and fertilizers. Jean-Baptiste Boussingault performed the first set of experiments in 1838 that showed nitrogen fixation with clover and pea. Between 1851 and 1855, he implemented a new set of experiments that were unsuccessful. The experiments carried out by Georges Ville at the same time showed a positive gain not only with legumes, but also with wheat, rye, and watercress. To kill the controversy, Ville performed new experiments under the control of a committee mandated by the French Academy (Dumas *et al*., 1855). Although this committee

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confirmed that Ville's observations were consistent with the conclusions drawn from his previous work, a number of questions were raised in the committee's report (Dumas *et al*., 1855) and Cloez (1855) highlighted a number of experimental difficulties casting doubt on the conclusions drawn. At about the same time, Gilbert, Lawes and Plugh conducted similar experiments at Rothamsted in England. The conclusions of these scientists, like those of Boussingault and Ville, were also censured by their contemporaries, in particular, the German scientist Liebig (Nutman, 1987).

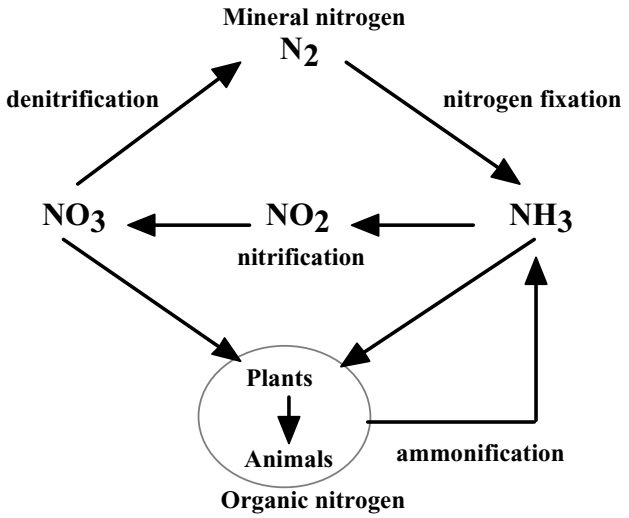

Figure 1. Schematic representation of the nitrogen cycle

Jodin, at the French Academy, reported the first observation of N_2 fixation by unknown microorganisms in a nutrient solution incubated under controlled conditions (Jodin, 1862). This observation was followed, 26 years later, by the isolation of a strain from root nodules by Beijerinck (1888). The strain, initially designated *Bacillus radicicola*, was later renamed *Bacterium radicicola*, and then as *Rhizobium leguminosarum* by Frank in 1890 (reviewed in Virtanen and Miettinen, 1963). A few years later, Winogradsky (1893) isolated the first anaerobic nitrogen fixer, *Clostridium pastorianum* (now *pasteurianum*) and, in 1901 and 1903, *Azotobacter* spp. were isolated by Beijerinck and Lipman (Table 1). Nitrogen fixation with blue-green algae (now classified as cyanobacteria) was also discovered during the 19th Century (see Chapter 12). However, as these algae were always associated with bacteria, it was only much later that their ability to fix nitrogen was confirmed (Drewes, 1928).

In 1883, the Danish scientist, Johann Kjeldahl, introduced an analytical method for the determination of total nitrogen and, one year later, the first digestion and

distillation equipment became available (produced by the C. Gerhardt Company). Berthelot (1885) first demonstrated chemical nitrogen fixation, by lightning for example, before turning his attention on nitrogen fixation by microscopic organisms in the soil, which he estimated would account for 15-to-30 kg of fixed N per ha.

By the end of the 19th Century, it was widely accepted that plants encourage the proliferation of microorganisms in the root zone. This led Lorenz Hiltner to define the rhizosphere as the soil immediately surrounding the roots under the influence of the plant (Starkey, 1958; Rovira, 1991).

2. NUTRITIONAL INTERACTIONS BETWEEN BACTERIA AND PLANTS

Following the initial discovery of *Clostridium* and *Azotobacter* an increasing number of nitrogen-fixing organisms were isolated (reviewed by Virtanen and Miettinen, 1963; Wilson, 1969; Postgate, 1982; Balandreau, 1983; Döbereiner and Pedrosa, 1987; Table 1). A dozen of genera had been discovered by 1969, including *Aerobacter* (*Klebsiella*), *Azotobacter*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Derxia*, *Spirillum*, and various photosynthetic bacteria and cyanobacteria (Stewart, 1969). Interestingly, *Spirillum* (*Azospirillum*) received little attention until the early 1970's (Döbereiner and Day, 1976). In fact, for more than 50 years after their initial discovery, *Azotobacter* and *Clostridium* were regarded as the only genera of bacteria capable of fixing nitrogen in the free-living state and *Nostoc* as the only nitrogen-fixing blue-green alga (Stewart, 1969; Wilson, 1969). But many soil bacteria were known to produce plant growth substances and to proliferate in the rhizosphere. Soon, "bacterization" was considered as a mean to benefit non-leguminous crops (Brown, 1974).

2.1. Azotobacter *and the Nitrogen-Fixation Potential of Soils*

In his volume on soil microbiology, which consists essentially of a compilation of his publications plus comments, Winogradsky (1949) expressed the view that *Azotobacter* was the only aerobic non-symbiotic bacterium able to fix nitrogen, with nitrogen fixation by other genera remaining doubtful. For Winogradsky, a key question was that of the role of *Azotobacter* in its natural environment. He differentiated "sugar *Azotobacter*" (grown in laboratories) from "soil *Azotobacter*" and considered that physiological experiments with pure cultures overfed with sugars provided the agrobiologist with no real insight into the role of *Azotobacter* in the soil. He developed several methods both for isolating *Azotobacter* and for estimating the density of this bacterium in soil, based on the use of either silica gel plates devoid of combined nitrogen or sifted soil to which mannitol or other carbon sources were added (see Pochon and Tchan, 1948). He proposed that the number of *Azotobacter* colonies was correlated with the nitrogen-fixation potential of the soil.

Table 1. Landmarks in nitrogen-fixation research with special reference to free-living, associative and endophytic nitrogen-fixing bacteria

Year	Event	Reference or citation
1838-1880	Experiments of Boussingault, Ville, Lawes and Gilbert, and others; controversy in the demonstration of nitrogen fixation by plants	Dumas et al, 1855[(a)]; Wilson, 1957; Nutman, 1987; Payne, 1990
1862	Jodin demonstrated nitrogen fixation by microorganisms in culture under controlled conditions	Jodin, 1862; Wilson, 1957
1856-1868	Reyset established the principle of the nitrogen cycle	Payne, 1990; Aubert, 1995
1877	Schlossing and Müntz discovered nitrification	Payne, 1990; Aubert, 1995
1883	Kjeldahl's method of total nitrogen determination	
1885	Berthelot observed nitrogen fixation in soil	Berthelot, 1885
1886	Gayon and Dupetit isolated the first pure culture of bacteria capable of denitrification	Payne, 1990; Aubert, 1995
1886-1888	Hellriegel and Wilfarth established nitrogen fixation by root nodules of Legumes	Wilson, 1957; Nutman, 1987
1888	Isolation of *Bacillus radicicola* [(b)]	Beijerinck, 1888
1890	Isolation of *Nitrosomonas* by Winogradsky, initially referred to as the "ferment nitrique"	Winogradsky, 1949; Payne, 1990
1893	Isolation of *Clostridium pasteurianum* [(c)]	Winogradsky, 1893
1901-1903	Isolation of *Azotobacter* spp. by Beijerinck and by Lipman	Virtanen and Miettinen, 1963
1904	Definition of the "rhizosphere" by Hiltner	Rovira, 1991
1925	Isolation of *Spirillum lipoferum* by Beijerinck	Becking, 1963; 1982
1927	First bacterization experiments in Soviet Union	Macura, 1966 [(d)]
1928	Isolation of *Aerobacter aerogenes* by Skinner	Virtanen and Miettinen, 1963
1928	Isolation of *Nostoc* and *Anabaena* by Drewes	Drewes, 1928; Chapter 12
1931	Discovery of production of phytohormones by bacteria	Boysen Jensen, 1931
1939	Isolation of *Beijerinckia* spp. by Starkey and De	Döbereiner and Pedrosa, 1987
1941	Application of N^{15} to Nitrogen fixation research	Burris and Miller, 1941
1949	Clark proposed the term of "rhizoplane" for the microbiology of root surface	Starkey, 1958; Rovira, 1991
1958	Isolation of *Bacillus polymyxa* by Hino and Wilson	Balandreau, 1983
1960	Nitrogenase activity is obtained in cell free extract of *C. pasteurianum* by Carnahan, Mortenson, Mower and Castle	Wilson, 1969
1961	Production of growth regulators by *Azotobacter*	Vancura, 1961
1966	Association *Azotobacter paspali -Paspalum notatum*	Döbereiner, 1974
1966	Acetylene reduction technique to assay nitrogenase activity by Schollhorn and Burris and by Dilworth	Hardy *et al*. 1968
1974	§ First international congress on nitrogen fixation, Pullman, Washington, USA, with the Döbereiner and Day paper on "Associative symbiosis in tropical grasses"	Newton and Nyman, 1976
1974-1978	Clarification of the taxonomic status of *Azospirillum* spp.	Tarrand *et al*., 1978

1979	‡ International workshop on associative N_2 fixation, São Paulo, Brazil	Vose and Ruschel, 1981
1980	Ausubel's and Haselkorn's groups used the high degree of conservation among *nif* genes for their detection in heterologous hosts by Southern hybridization	Elmerich, 1991
1981	¶ First "*Azospirillum* workshop", Bayreuth, Germany	Klingmüller, 1982
1986	Isolation of *Herbaspirillum seropedicae*	Döbereiner, 1992
1986	Isolation of nitrogen-fixing rod from Kallar grass (later identified as *Azoarcus* spp.)	Reinhold *et al*., 1986
1987	Nitrogen fixation in *Pseudomonas stutzeri*	Krotzky and Werner, 1987
1988	Isolation of (*Glucon-)Acetobacter diazotrophicus*	Döbereiner, 1992
1992	Development of the N_2-fixing endophytes concept	Döbereiner, 1992
1994	Discovery of nitrogen fixation in *Burkholderia* associated with rice	Tran *et al*., 1994; Chapter 2
1995	International Symposium on sustainable agriculture, Rio de Janeiro, Brazil, organized by Franco and Boddey in honour of the 71st birthday of Johanna Döbereiner	Special issue of Soil Biol. Biochem., 1997, 29, N°5/6
2001	Non-culturable *Burkholderia* endophyte	Minerdi *et al*. 2001
2001	Development of the β-rhizobia concept: some β-Proteobacteria can nodulate legumes[(e)]	Moulin *et al*. 2001; see Chapter 2
2002	Genome projects: *Azotobacter*, *Herbaspirillum*	Kennedy, Pedrosa *et al*

(a) *This ref. corresponds to the report presented to the French Academy by the committee members who evaluated the experiments performed by Ville;* ***(b)*** Rhizobium leguminosarum*;* ***(c)*** *In his initial publication of 1893, Winogradsky isolated a mixture of 3 bacilli; in 1894, he successfully isolated the nitrogen-fixing agent in pure culture in anaerobic conditions; and the name* Clostridium pastorianum *appeared only in 1895;* ***(d)*** *J. Macura presented his general report at the Soil Microbiology Colloquium, devoted essentially to "bacterization", organized by J. Pochon in Feb. 1966.* ***(e)*** *The ability to establish a symbiosis with legumes is found outside the α Proteobacteria and among β Proteobacteria in the Burkholderiales:* Burkholderia *and* Ralstonia. ***§‡ ¶****- The numerous International Congresses covering different aspect of nitrogen fixation cannot be cited here, but these three series have been of particular importance for the field.* ***§*** *1974 saw the first of a series of international congresses set up by W. E. Newton, covering chemistry, biochemistry, genetics, ecology and agricultural aspects of nitrogen fixation; the following were in Salamanca, Spain (1976), Madison, USA (1978), Canberra, Australia (1980), Noordwijkerhout, The Netherlands (1983), Corvallis, USA (1985), Cologne, Germany (1987), Knoxville, USA (1990), Cancun, Mexico (1992), St Petersburg, Russia (1995), Paris, France (1997), Foz do Iguaçu, Brazil (1999), Hamilton, Canada (2001) and Beijing, China (2004).* ***‡*** *After the 1979 workshop in Brazil, the symposia on "Nitrogen Fixation with Non legumes" were held successively in Canada (1982), Finland (1984), Brazil (1987), Italy (1990), Egypt (1993), Pakistan (1996), Australia (1999) and Belgium (2002).* ***¶*** *The first four workshops were organized by W. Klingmüller in Bayreuth, Germany in 1981, 1983, 1985 and 1987, with I. Fendrik and associates continuing the tradition in Hanover, Germany (1991) and Hungary (1994). The* Azospirillum *workshops are now a part of the "Nitrogen Fixation with Non legumes" series. Congresses on "photosynthetic prokaryotes", not mentioned here, are also regularly held and are of importance for the research on cyanobacteria.*

2.2. Contribution of Free-Living Nitrogen Fixers to Soil Fertility

With the emergence of ^{15}N tracer techniques (Burris and Miller, 1941), the relative contribution to soil fertility by free-living nitrogen-fixing organisms continued to stimulate considerable interest (Delwiche and Wijler, 1956; Chang and Knowles, 1965; Stewart, 1969). *Azotobacter* decreased in importance in the eyes of researchers (Starkey, 1958) as new nitrogen-fixing species were confirmed by the isotopic technique (Wilson, 1969).

Beijerinckia was demonstrated to be of importance in soils following the discovery that *Azotobacter* distribution is limited to neutral soils, whereas *Beijerinckia* predominates in tropical acid soils (Dommergues and Mutaftschiev, 1965; Döbereiner, 1974; Döbereiner and Pedrosa, 1987). Similarly, anaerobic nitrogen fixation by *C. pasteurianum* in soils and aerobic nitrogen fixation by genera other than *Azotobacter* was observed in the soils of Quebec (Chang and Knowles, 1965). The addition of soluble organic substrates to soils greatly increased the rate of nitrogen fixation. In natural conditions, free-living heterotrophs in the soils have been shown to fix insignificant quantities of nitrogen unless organic substrates, such as grass cuttings, straw or other plant residues, are available (Delwiche and Wijler, 1956).

2.3. Bacterization

Plants were first inoculated with bacterial preparations in 1895, when Nobbe and Hiltner reported the benefit of inoculating legume seeds with rhizobia (Subba Rao, 1982). This development constituted the birth of the commercial inoculant industry, with the establishment in 1898 of Nitragin, a company that still produces rhizobial inoculants.

Inoculation was later extended to non-leguminous crops, such as cereals and vegetables. Shortly after the discovery of *Azotobacter*, the effects of inoculating the soil with this bacterium were investigated with a view to improving soil nitrogen balance and plant growth. Further experiments followed in which seeds or roots were directly inoculated with *Azotobacter*. The term "bacterization" was coined in 1926 and field inoculations with "azotobacterin" began in the Soviet Union shortly afterwards (see Macura, 1966). Russia was very active in this field of research, because Russian soils contained large numbers of *Azotobacter*. By 1958, about 10 million ha in Russia were treated with preparations of either *Azotobacter chroococcum* or *Bacillus megaterium* (Brown, 1974; Rovira, 1991) with *Bacillus* used for organic phosphate mineralization (phosphobacterin).

The reported results of bacterization in Russia generated strong controversy due to claims of high yield increases that were not reproduced in other parts of the world. However, further experiments confirmed some increase in yield for various crops. Moreover, changes were also reported in the general growth of the plants, for example, early flowering in tomato and wheat (Michoustine, 1966; Dénarié and Blachère, 1966; Brown 1974; Rovira, 1991). Also, other bacterial species, which may have beneficial effects, were detected in the plant rhizosphere (Rivière, 1963; Dénarié and Blachère, 1966; Brown 1974; Döbereiner and Day, 1976).

2.4. Role of the Bacterial Inoculant in the Bacterization Process

A prerequisite for successful bacterization is the survival of the inoculum and its multiplication in the rhizosphere. However, *Azotobacter* is often described as a fragile organism, highly sensitive to pH variations, with a low survival rate in the soil (Pochon and Tchan, 1948; Postgate, 1981; Döbereiner and Pedrosa, 1987). Many researchers have reported rapid declines in the number of *Azotobacter* after inoculation and a lack of rhizoplane (root surface) colonization (Jackson and Brown, 1966; Michoustine, 1966; Subba Rao, 1982; Kloepper, 1994).

The means by which the plants benefit from the inoculation was unclear. Different hypothesis were proposed to explain the benefit observed. These included: (i) changes in the rhizosphere microbial population; (ii) production of growth regulators (phytohormones) that stimulated the plant development; and (iii) disease suppression (Brown, 1974; Subba Rao, 1982; Rovira, 1991). Bacteria are known to produce growth regulators (Boysen Jensen, 1931; Vancura, 1961). *Azotobacter* cultures produce gibberellic acid and indole-3-acetic acid (Vancura, 1961; Brown, 1974). Rivière (1963) noted that a high percentage of bacterial strains isolated from the rhizosphere of wheat produced phytohormones. The list of phytohormone producers among soil bacteria and plant pathogens and the variety of the compounds synthesized is very large (see Chapter 6, this volume). Furthermore, nitrogen fixation and phosphorus mineralization were not considered to play a major role in the efficient application of "azotobacterin" and "phosphobacterin" (Brown, 1974; Subba Rao, 1982; Rovira, 1991).

2.5. The Rhizosphere Effect

The work of Hiltner in particular increased our understanding of the differences between the bulk soil and the rhizosphere soil. The microbial population is dense around plant roots. Plants affect microbe development and, in turn, the plant is affected by the activity of the microbes in the rhizosphere (Starkey, 1958). From 1950 onwards, studies of the composition of root exudates increased (reviewed by Starkey, 1958; Rovira, 1962). These analyses demonstrated that root exudates provide a source of nutrients for the soil microflora, favouring the proliferation of certain microorganisms in the rhizosphere and preventing others (Rovira, 1962). Clark defined the rhizoplane in 1949 (Starkey, 1958; Rovira, 1991) and the term "rhizodeposition" was later coined to account for the carbon loss by the plant that generates the rhizosphere effect (Lynch and Whipps, 1991). An important feature of the root exudates is their high C/N ratio, which may promote enrichment in nitrogen-fixing bacteria in the rhizosphere (Döbereiner, 1974).

The term "rhizobacteria" is now currently used for the bacteria that colonize the rhizosphere. Rhizobacteria with beneficial effects on plant development (involving growth stimulation or disease prevention/suppression) are referred to as plant growth-promoting rhizobacteria or PGPR (Kloepper and Beauchamp, 1992; Kloepper, 1994).

3. ASSOCIATIVE NITROGEN-FIXING BACTERIA

The terms "associative symbiosis", "rhizocoenoses", and "associative nitrogen fixation" have all been used to describe the interaction between *Azospirillum* and other rhizosphere bacteria and their host plants. None of these is fully satisfactory as a generic term. The process and the degree of interaction between the bacteria and the plant may differ between species; there are no differentiated structures on the roots induced by the bacteria; the extent of rhizoplane colonization is not always well defined; and the benefit of the association has often been challenged. Thus, soil nitrogen-fixing bacteria that can be found in close association with the root of grasses are usually designated as "associative nitrogen-fixing bacteria". Due to their growth-promoting effect, they are also referred to as nitrogen-fixing PGPR.

3.1. Evidence for Nitrogen Fixation in non-Legume Cropping Systems

Early evidence for non-symbiotic nitrogen fixation (reviewed by van Berkum and Bohlool, 1980) was provided by studies of the N balance in various ecosystems, such as salt marshes, fallow fields, and pasture fields. Crops, such as sugarcane in the tropics, and wetland rice in Asia, together with certain cereal fields in Canada and fallow fields in UK, shared the common characteristic of receiving no fertilizers over several centuries, and were all thought to benefit from nitrogen fixation by some means (van Berkum and Bohlool, 1980; Boddey and Döbereiner, 1982).

The advent of new techniques, based on acetylene reduction by nitrogenase, made it easier to estimate nitrogen fixation in natural ecosystems (Hardy *et al*., 1968; 1973). A critical review of the various techniques available for use with bacterial cultures, legumes, and non-leguminous plants can be found in the manual edited by Bergersen (1980). The determination of acetylene reduction either on excised roots or on plant soil cores was a source of some controversy because it did not reflect the actual rate of nitrogen fixation in intact plants in their natural environments (Hirota *et al*., 1978; van Berkum and Bohlool, 1980). In most cases, there was a considerable time lag before nitrogenase activity became detectable, and large variations between samples were observed. Balandreau *et al.* (1974) determined *in situ* acetylene reduction for a grass (*Panicum maximun*), rice, and peanut and not only confirmed the existence of non-symbiotic nitrogen fixation, but also demonstrated plant-specific diurnal variations in nitrogen-fixation rates.

In the preface of the book devoted to "Nitrogen Fixing Bacteria in Non-leguminous Plants" (Döbereiner and Pedrosa, 1987), Johanna Döbereiner describes her sabbatical leave at Rothamsted (UK) in 1970-1971. It was during her stay that she, together with other members of the "Grass-N_2-Fixation-Club", found acetylene reduction with roots of sugarcane and several tropical grasses, including *Paspalum notatum* (Döbereiner *et al*., 1972a). The discovery of *Azotobacter paspali* was an important step into associative nitrogen fixation. This species of *Azotobacter* is specific for *Paspalum notatum* cv. batatais and estimates of N_2 fixation with different soil cores ranged from 15-90 kg N/ha/year (Döbereiner *et al.*, 1972b). The ^{15}N isotope-dilution method showed that 10% of the total-N accumulated in *Paspalum notatum* originated from biological N_2 fixation (Boddey *et al*., 1983).

However, Brown (1976) observed that acetylene reduction was not always associated with the presence of *A. paspali* on the roots and she claimed that *A. paspali* improved the growth of *P. notatum* primarily by producing phytohormones rather than by N_2 fixation. This nitrogen fixation *versus* phytohormones production appears as a recurrent theme in the history of associative nitrogen-fixation research.

Boddey and Döbereiner (1982) reviewed early reports of N_2 fixation in rice, which showed up to 20-30 % of total-N in rice plants originates from biological N_2 fixation (Ventura and Watanabe, 1982; Watanabe and Roger, 1984). However, as the wetland rice microflora is highly complex, it was difficult to estimate the contribution of the heterotrophic bacterial population to N_2 fixation. Sano *et al*. (1981) measured the acetylene-reduction rates of various rice cultivars *in situ* and showed seasonal and diurnal variation as well as cultivar-dependent N_2 fixation.

3.2. Microbiology of the Association

3.2.1. Identification of the Nitrogen-Fixing Bacteria

In a review paper, Johanna Döbereiner listed her "ten recommendations" for the identification of root-associated diazotrophs (Döbereiner, 1989). In addition, the development of both numerical and molecular-taxonomy techniques, plus phylogenetic analyses, has greatly advanced the identification of the putative nitrogen-fixing isolates (Rennie, 1980; Balandreau, 1983; Hartmann *et al*., 2000; Roselló-Mora and Amann, 2001). Indeed, with time, many isolates have been renamed and reclassified in new genera (Young, 1992). Tools and strategies for the identification of bacterial isolates and for the *in situ* localization of these bacteria in the rhizosphere or within the plant tissues are detailed in Chapter 2 of this volume.

Determination of the nitrogen-fixation capacity of the bacterial isolates is often a critical step. In most cases, growth in N-free solid, semi-solid or liquid media is insufficient proof of nitrogen-fixing activity. Physiological conditions compatible with nitrogenase activity and the detection of this activity by the acetylene-reduction test may also be ineffectual or inconclusive (Postgate, 1981). Therefore, a molecular demonstration of the presence of the *nif* genes in the genome of the putative nitrogen fixer is generally considered as the reliable indicator. After the identification and cloning of the nitrogenase structural genes, *nifHDK*, and related genes, Southern hybridization experiments were performed to identify *nif* genes in a large number of Eubacteria and Archaea (Table 1, reviewed by Elmerich, 1991). Nowadays, the polymerase chain reaction (PCR) amplification with *nif* specific oligonucleotides probes, such as "*nifH* universal primers", is preferred (Zehr and McReynolds, 1989). This technique can be applied to the DNA of pure bacterial cultures, but it can also be used to follow the fate and distribution of nitrogen fixers by amplifying *nif* DNA fragments from DNA extracts from environmental samples (Rosado *et al.*, 1998; Hamelin *et al*, 2002; see Chapter 2).

3.2.2. Old and New Nitrogen-Fixing Bacteria

By the end of the 1980's, the presence of various species of *Azotobacter, Bacillus*, *Beijerinckia, Derxia, Enterobacteriaceae* (*Klebsiella, Enterobacter, Pantoae*),

Pseudomonas, and *Pseudomonas*-like bacteria was well established in the rhizosphere of cereal crops, weeds, and sugarcane (Rennie, 1980; Balandreau; 1983; Bally *et al.*, 1983; Haahtela *et al*, 1983b; Ladha *et al.*, 1983; Seldin *et al.*, 1984; Young, 1992). For nitrogen-fixing *Pseudomonas,* most of the initial reports dealt with bacteria that have since been reclassified to other genera (see Chapter 3), however, Vermeiren *et al*. (1999) confirmed the identity of the isolates classified as *Pseudomonas* by Haahtela *et al*. (1983a) and Krotzky and Werner (1987) (see Chapter 3). Johanna Döbereiner was responsible for discovering most of the new nitrogen-fixing species that were isolated from close association with the roots of various forage grasses, sugarcane, and maize (Table 1). In 1961, she found *Beijerinckia* associated with sugarcane roots (Döbereiner, 1961); in 1966, she isolated *Azotobacter paspali* from the grass growing in front of her laboratory (Döbereiner, 1974); and she then isolated several species of *Azospirillum* (Chapter 2), followed by *Herbaspirillum* in 1986, and of *Gluconacetobacter* in 1988 (Table 1; see Section 4.2).

Little attention was paid to the spirillum-like bacteria isolated by Beijerinck in 1923 and rediscovered by Becking (1963; 1982) until Döbereiner and Day (1976) described the association of these bacteria with grasses and many cereal crops. These bacteria were eventually assigned to a new genus, called *Azospirillum* (Tarrand *et al*., 1978), and the number of reports dealing with these bacteria increased rapidly thereafter. Although this volume extends well beyond *Azospirillum*, much of the information published on this genus can be found in Chapters 2-8. *Azospirillum* species display an extremely wide ecological distribution and are associated with a large diversity of plants (van Berkum and Bohlool, 1980). Seven species are known (Chapter 2) and many aspects of their physiology and genetics have been reviewed (Eskew *et al*, 1977; van Berkum and Bohlool, 1980; Patriquin *et al*., 1983; Döbereiner and Pedrosa, 1987; Elmerich *et al*., 1992; 1997; Okon, 1994; 1985; Bashan and Levanony, 1990; Costacurta and Vanderleyden, 1995; Steenhoudt and Vanderleyden, 2000), including *nif* genetics (Chapter 3), colonization of the root system (Chapters 4 and 5), phytohormone production (Chapter 6), and the plant response to inoculation (Chapter 7). These bacteria are also known to produce siderophores and bacteriocins, which may serve as biocontrol agents in the competition with other members of the soil microflora (see Chapter 8).

The microbiology of rice, maize, coffee, sugarcane, pineapple, sorghum, and Kallar grass also resulted in the characterization of new species of *Alcaligenes*, *Azoarcus*, *Burkholderia*, *Campylobacter*, *Gluconacetobacter*, *Herbaspirillum,* and *Paenibacillus*, many of which are probably endophytes (see Section 4.2 and Chapters 2, 9, 10, and 13).

Flooded rice fields are an important source of methane emissions into the atmosphere (Liesack *et al*., 2000). Methane is produced as a result of a complex interaction between bacteria in anoxic soil, the oxic interface, and the rice rhizosphere (Watanabe and Roger, 1984). The bulk soil is considered as an anoxic compartment that favours the proliferation of fermentative bacteria (*Clostridium* spp.), methanogenic Archaea, and sulfate-reducing bacteria (*Desulfovibrio*),

whereas methane-oxidizing bacteria are present at the aerobic interface. Members of all of these groups are known to fix N_2 (Postgate, 1981; Young, 1992).

3.3. Plant-Growth Promotion

Efficient nitrogen-fixing associations require an adequate supply of substrates from the host plant, appropriate environmental conditions to support nitrogen fixation by the associated bacteria, and transfer of the fixed nitrogen to the host plant (Klucas, 1991). In 1975, von Bülow and Döbereiner reported high rates of nitrogen fixation by *Azospirillum* in association with maize. These findings generated a considerable long-standing controversy, even though experiments with the ^{15}N isotope dilution technique confirmed that, in some cases, biological nitrogen fixation could account for several percent of the total nitrogen in the plant (Boddey and Döbereiner, 1982; Klucas, 1991). The controversy remained until 1994, when Okon and Labandera-Gonzales published a compilation analysis of field trials. This survey of 20 years of field inoculation worldwide concluded that significant (5-30%) increases in yields could be achieved by inoculation with *Azospirillum*. These crop-yield increases were more marked when the use of chemical fertilizer was low. Yield increases result from better development of the root system, which correlates with an increase in the rate of water and mineral uptake by roots. *Azospirillum* possesses several pathways for IAA synthesis and also produces gibberellins. The plant growth-promoting effect of this genus is currently attributed to production of indole-3-acetic acid (IAA) and other phytohormones (see Chapters 6 and 7). The nitrogen-fixing capacity of these bacteria is thought to contribute little to plant growth.

4. DISCOVERY OF NITROGEN-FIXING ENDOPHYTES

The presence of bacteria resident within the tissues of healthy plants was first reported as early as 1926 (Starkey 1958; Hallmann *et al*, 1997). The names "endorhizospheric" and "endophyte" are used to describe this particular type of bacteria-plant association that does not induce disease symptoms (You and Zhou, 1988; Döbereiner, 1992; Reinhold-Hurek and Hurek, 1998). The systematic isolation of nitrogen-fixing bacteria, which belonged to bacterial species that did not survive in the soil, from externally sterilized root and stem samples led Döbereiner and co-workers to define a novel type of nitrogen-fixing bacterium-plant interaction involving nitrogen-fixing endophytes (Döbereiner, 1992; Döbereiner *et al.*, 1993).

4.1. Azospirillum*: a Root Surface Colonizer or a Facultative Endophyte?*

Azospirillum species are indigenous soil bacteria and common root-associated diazotrophs, essentially located on the surface of the root. They are connected to the root surface by fibrillar material and are sometimes found in the superficial layers of the root cortex (Bashan and Levanony, 1990). Indeed, most of the *Azospirillum* isolates have been obtained from surface-sterilized root samples, suggesting that a proportion of these cells are protected from sterilizing agents and

are present in root tissues (Döbereiner and Day, 1976; Umali-Garcia *et al*., 1980; Patriquin *et al.*, 1983; Okon, 1985). However, it has also been argued that some bacteria may survive the sterilisation step and then could proliferate in the damaged tissues (Umali-Garcia *et al.*, 1980; Hallman *et al*. 1997). It is now widely accepted that the extent of internal colonization may depend on the bacterial strain, the plant species, and other unidentified factors (Bashan and Levanony, 1990). As reported in Chapter 2, *A. brasilense* Sp7 is found preferentially on the root surface, whereas strain Sp245 is found in the intercellular spaces of the root epidermis (Rothballer *et al*., 2003).

4.2. Endophytes of Sugarcane, Rice, Maize, and Kallar Grass

4.2.1. Discovery of Herbaspirillum *and* Gluconacetobacter

Sugarcane and rice both give high yields with little or no addition of chemical fertilizers (van Berkum and Bohlool, 1980). Two novel nitrogen-fixing species, *Herbaspirillum* and *Gluconacetobacter*, are commonly found with sugarcane in Brazil. Both are acid tolerant and capable of growth and nitrogen fixation in media containing up to 10% sucrose. As these bacteria do not persist in soil but can be isolated from roots, stems, and leaves and are also found in xylem vessels, they were considered as nitrogen-fixing endophytes (Döbereiner 1992).

Herbaspirillum was initially isolated from maize, in 1986, as a novel *Azospirillum* species, named as *A. seropedicae*, but it rapidly became apparent that this bacterium belonged to the β-subgroup of Proteobacteria and constituted a new species. It was renamed as *Herbaspirillum seropedicae* (Döbereiner and Pedrosa, 1987; Döbereiner, 1992). This newly defined species was found to be very closely related to a mild pathogen of sugarcane, identified first as *Pseudomonas rubrisubalbicans,* another nitrogen-fixing species. Further analysis revealed that both *H. seropedicae* and *P. rubrisubalbicans* were phylogenetically closely related and that both gave disease symptoms on *Sorghum* and *Pennisetum* (Döbereiner, 1992). *H. seropedicae* is also found in roots of rice, sorghum and forage grasses (Döbereiner et *al*., 1993).

Gluconacetobacter was initially identified as an acid-tolerant bacterium that produced an orange-brown pigment and displayed general properties similar to those of *Acetobacter* spp. It was first named *Saccharobacter nitrocaptans*, then *Acetobacter nitrocaptans*, followed by *Acetobacter diazotrophicus* (Döbereiner, 1992), and is currently classified as *Gluconacetobacter diazotrophicus* (see Chapter 2). *G. diazotrophicus* resides in many sugarcane cultivars, and in sweet potato, and new species of *Gluconacetobacter* have been isolated from coffee and pineapple. *Gluconacetobacter* is also present in spores of vesicular-arbuscular mycorrhizal fungi (VAM). The association of VAM with the roots of vascular plants increases the absorption of nutrients, phosphorus in particular, and occurs in most of the important agricultural species. The inoculation of plants with the VAM fungi containing *G. diazotrophicus* increases the translocation of the bacterium in the aerial parts of the plant (Döbereiner *et al.*, 1993).

Since the discovery of *G. diazotrophicus* and *H. seropedicae,* the phylogeny, physiology, and genetics of these bacteria and their colonization of sugarcane have been extensively studied (see Chapters 2, 3 and 10). The *H. seropedicae* genome sequencing is close to completion (Chapter 3). *G. diazotrophicus* has been reported to contribute significant fixed nitrogen to sugarcane and *Herbaspirillum* is also thought to increase productivity (Chapter 10).

4.2.2. Endophytes of Kallar Grass and Rice: Azoarcus *and other Species*

Vast areas of land previously suitable for cultivation are now affected by increasing salt concentrations. Kallar grass is a salt-tolerant plant, common in salt marshes in Pakistan, which can be used for land reclamation and biomass production (Malik *et al*., 1986). Kallar grass-associated nitrogen fixation has been confirmed by the ^{15}N dilution technique and several nitrogen fixers, including both *Klebsiella* and *Azospirillum* species, have been isolated from the roots of this plant (Malik *et al*., 1986; 1991). In Pakistan, Reinhold *et al*. (1986) isolated several nitrogen-fixing species from Kallar grass; a new halotolerant *Azospirillum* species (later identified as *A. halopraeferens*) was found on the rhizoplane, whereas Gram-negative rods (later identified as *Azoarcus*) were found to colonize the root interior (Reinhold-Hurek and Hurek, 1998). In other parts of the world, including salt marshes in the US in particular, analysis of the nitrogen-fixing communities associated with cordgrass (*Spartina alterniflora*) revealed a large biodiversity, including γ Proteobacteria and anaerobic diazotrophs, with a majority of as yet unidentified species (Lovell *et al*., 2000).

Azoarcus can infect the root system and the xylem vessels of Kallar grass and rice (Hurek *et al.*, 1991; Reinhold-Hurek and Hurek, 1998). In the free-living state, either at very low oxygen tension or in association with a fungus, the bacterium can differentiate into a hyper-induced state for nitrogen fixation and develop an intracytoplasmic membrane system known as diazosomes (Hurek *et al*., 1995). *Azoarcus* sp. strain BH72 expresses structural genes that encode nitrogenase in association with the host plant and recent data have shown that, in an unculturable state, this bacterium can supply the plant with fixed-nitrogen (Hurek *et al.*, 2002; see also volume 4 of this series *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment*). The genetics and regulation of nitrogen fixation have been studied in *Azoarcus* sp. BH72 in particular (see Chapters 3 and 9) and into the process of association with the host is documented in Chapter 9.

Rice microbiology has been extensively studied in China, where 26% of the cultivable land is under rice (You *et al*., 1991). In the early 1980's, C. B. You's team isolated strains from rice roots that they identified as *Alcaligenes faecalis* and showed that this species was common in paddy soil (You and Zhou, 1988). These bacteria were described as endorhizospheric. They colonized the intercellular spaces of the root epidermis and some penetrated the cell wall. Rice callus inoculated with strain A15 was shown, by means of the ^{15}N tracer technique, to fix nitrogen (You and Zhou, 1988). Strain A15 was later reassigned to the species *Pseudomonas stutzeri* (Vermeiren *et al*., 1999). The *nif* genetics of *P. stutzeri* is described in Chapter 3.

4.3. Emerging Systems

Although nitrogen-fixing *Klebsiella* strains have been isolated from some plants, including rice, there are few reports concerning the ability of these strains to colonize their host (Balandreau, 1983; Haahtela *et al*., 1983b; Ladha *et al*., 1983; You *et al*. 1991). A *K. pneumoniae* strain was recently isolated from maize (Chelius and Triplett, 2000) and was shown to be an endophyte and to promote maize growth. The importance and limits of this system, and those of other endophytes, like *Gluconacetobacter*, *Herbaspirillum*, and *Azoarcus*, are discussed in Chapter 13.

In cropping systems involving rotation that includes legumes, several authors have reported the association of rhizobia with rice and maize. Yanni *et al.* (1997) described a *Rhizobium leguminosarum* endophyte of rice, whereas Gutiérrez-Zamora and Martínez-Romero (2001) reported the association of maize with *R. etli*.

Structural genes encoding nitrogenase have been identified in a non-culturable *Burkholderia* sp. associated with VAM fungi (Minerdi *et al*., 2001). This observation confirms that VAM fungi can harbour putative nitrogen-fixers and raises the question of the importance, diversity, and role in plant nutrition of non-culturable bacterial endophytes.

5. CYANOBACTERIAL ASSOCIATIONS

A historical perspective of the main steps in the discovery of cyanobacteria can be found in Chapter 12. Regarded as algae for a long time because they performed plant-type photosynthesis, they were commonly named blue-green algae or cyanophytes. Cyanobacteria are now classified within the Prokaryote kingdom in the Eubacteria (Stanier and Cohen-Bazire, 1977).

Cyanobacteria perform oxygenic photosynthesis and play an important role in maintaining the CO_2/O_2 balance on our planet (Kasting and Siefert, 2002). Many species are capable of nitrogen fixation. The most important group of N_2-fixing cyanobacteria consists of filamentous strains that differentiate to generate heterocysts. *Nostoc* and *Anabaena* are the best-known representatives of this group. Heterocysts are the site of nitrogen fixation and these highly specialized cells differentiate within the filaments according to a complex regulatory network, as described in Chapter 11.

Cyanobacteria colonize most aquatic (fresh water and sea) and terrestrial ecosystems and are found in symbiotic association with many plants, including lichens, liverworts, and cycads, *Gunnera* (an angiosperm), and the aquatic fern *Azolla* (see Chapter, 12). *Azolla* is often considered to be a weed that can develop uncontrollably in ponds. However, in Asia, in Vietnam and southern China in particular, the use of *Azolla* as a green manure is an ancient practice (Peters, 1977).

Cyanobacteria play a major role in rice cultivation (Peters, 1977; Roger and Kulasooriya, 1980; Subba Rao, 1982). Cyanobacteria contribute a mean of 30 kg N per ha to a crop, whereas the heterotrophic flora contribute a mean of 7 kg N per ha (Ladha *et al*., 1997). Ladha *et al*. (1997) also reported that the use of *Azolla* or

semi-aquatic legumes, such as *Sesbania* and *Aeschynomene*, as green manures results in the production of about 60 kg N per ha.

Watanabe *et al*. (1951) investigated the cyanobacteria proliferating in rice fields in Asia. They collected many samples and identified 13 different nitrogen-fixing species. They reported, in particular, an increase of 15-25% in rice yield after inoculation with *Tolypotrix tenuis*. Such inoculation practices (referred to as algalization) have been extended to many rice-producing countries (Subba Rao, 1982). Roger and Kulasooriya (1980) carried out an extensive literature compilation of this practice, taking into account both the biotic and abiotic factors that may limit the formation of blooms after inoculation. The colonization of rice by epiphytic or endophytic cyanobacteria has also been reported (Roger and Kulasooriya, 1980; Watanabe and Roger, 1984). Additional information the biology of the water fern *Azolla* and on rice and wheat colonization by cyanobacteria is found in Chapter 12.

6. CONCLUDING REMARKS

Nitrogen fixation by cereal crops is often considered negligible in comparison with that observed in legumes and actinorhizal plants. However, most of the available cultivated land is used for cereal and forage crop production. Rice is the major food for more than a third of the world's population. There is an urgent need to increase food production to meet the demands of an expanding world population. Exploration of the potential role of free-living nitrogen-fixing heterotrophic organisms in increasing soil fertility and providing plants with nutrients began some 80 years ago. It took about 50 years before it was recognized (Döbereiner and Day, 1976) and accepted (Okon and Labandera-Gonzales, 1994) that the association of nitrogen-fixing bacteria with graminaceous plants was beneficial. Indeed, it is still argued that nitrogen fixation has little to do with plant-growth promotion and that phytohormone production is responsible for the observed benefits (Brown, 1974; Bashan and Levanony, 1990). Increases in yield have been consistently reported from the early bacterization experiments with *Azotobacter* (Brown, 1974), followed by the use of *Azospirillum* (Boddey and Döbereiner, 1982), the role of bacterial endophytes (Döbereiner *et al.*, 1993) and the use of cyanobacteria (Roger and Kulasooriya, 1980).

The development of molecular tools for phylogeny analysis in the last decade has led to the discovery of an increasing number of new species. The *Burkholderia* genus now includes 39 species and new *Azospirillum, Gluconacetobacter*, *Herbaspirillum*, *Paenibacillus, Klebsiella,* and *Pantoae* species have been described in association with plants. New *in situ* localization tools now make it possible to define precisely the relationship of these organisms to the host plant (Hartmann *et al*., 2000). In terms of physiology and molecular genetics, tremendous progress has been made in advancing our knowledge of the regulation of the *nif* genes and of the factors controlling the interaction with the host plant, both for nitrogen-fixing heterotrophs and for cyanobacteria. Following the identification of nitrogen-fixing endophytes for the major cereal crops and the demonstration that some ecosystems benefit from nitrogen fixation, *e.g*., sugar cane, rice, and maize, it appears that

research into associative and endophytic nitrogen fixation and the associations between plants and cyanobacteria has a promising future.

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Chapter 2

MOLECULAR PHYLOGENY AND ECOLOGY OF ROOT ASSOCIATED DIAZOTROPHIC α- AND β-PROTEOBACTERIA

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1. INTRODUCTION

The knowledge of the natural diversity of root-associated diazotrophs is becoming increasingly complex and fascinating as more and more species of plant-associated diazotrophs are successfully isolated and cultivated from a wide variety of plants, especially from subtropical and tropical regions. The application of molecular genetic detection and identification methods greatly aids in clarifying the phylogenetic relationships of these bacteria. It is generally accepted that only a combination of methods, including classical cultivation techniques and cultivation-independent techniques, enable a comprehensive insight into the bacterial diversity in environmental habitats (Hartmann *et al*., 1997; Liesack *et al*., 1997). It has been repeatedly demonstrated that a high bacterial diversity can be revealed using molecular techniques that directly target either the diversity of the 16S-rDNA (Amann *et al*., 1995), the most used genetic marker for molecular phylogenetic studies, or the diversity of the *nif* genes (Ueda *et al.*, 1995). Concerning the rhizosphere environment, the degree of cultivability is assumed to be high due to the good growth conditions for microbes in the root environment, however, for the grass endophytic *Azoarcus* spp., an unculturable state has recently been demonstrated (Hurek *et al*., 2002).

The ribosomal RNA genes of bacteria, especially those for 16S- and 23S-rRNA, are excellent molecular markers for phylogenetic studies because of their functionally constancy, their ubiquitous distribution, and elements rising from

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highly conserved-to-highly variable regions within the sequence (Ludwig *et al.*, 1998). This molecular phylogenetic approach can be used to identify pure isolates and to assess the diversity of complex communities. Since powerful amplification and sequencing techniques became available in the last decade, more than 16,000 complete and partial sequences have been deposited in data banks like NCBI. Modern software, *e.g.*, the widely used software package ARB, has also been developed to handle all these data for phylogenetic analysis (Ludwig and Strunk, www.arb-home.de; Ludwig *et al.*, 2004). In addition, sophisticated software packages are available not only to use this information for phylogenetic evaluation but also for the development of discriminative oligonucleotide probes for diagnostic purposes. In cases of very close phylogenetic relationship, the higher information content of either the 23S-rDNA or the intergenic spacer region of the rDNA-operon (IGS-region; Tan *et al*., 2001) offers additional very valuable molecular markers for phylogenetic studies and strain differentiation.

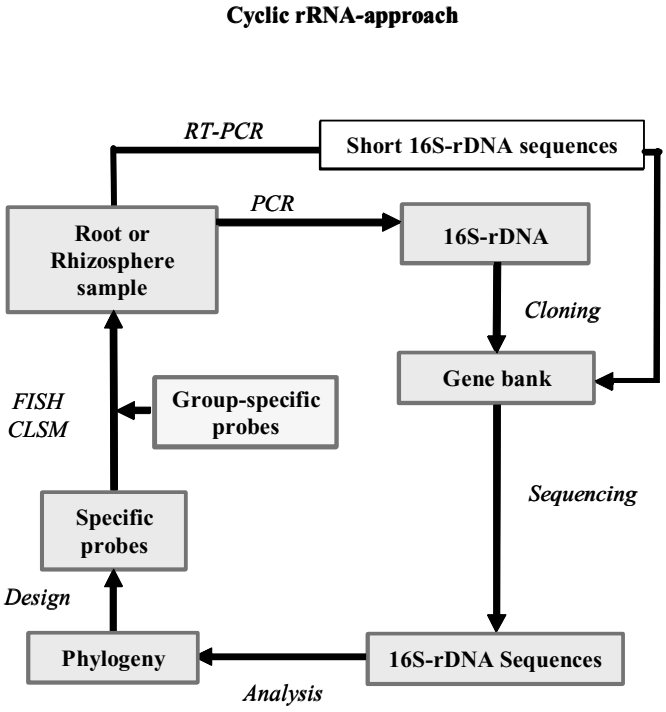


Figure 1. Cyclic rRNA-approach
Schematic representation of the different steps used for identification and in situ *localization of associated/endophytic nitrogen-fixing bacteria.*

The development of 16S-rRNA-targeting fluorescence-labeled phylogenetic oligonucleotide probes enable us to identify active bacterial cells in their natural

habitat by using the fluorescence *in situ* hybridization (FISH) technique (Amann *et al*., 1995), thereby closing the so-called "cyclic rRNA-approach" (Figure 1).

2. TOOLS FOR MOLECULAR PHYLOGENY AND *IN SITU* LOCALIZATION OF BACTERIAL ISOLATES AND COMMUNITIES

2.1. Use of 16S-rDNA as Phylogenetic Marker

Due to its wide and successful use as a phylogenetic marker, the 16S-rDNA provides an indispensable tool for the classification and identification of bacteria. In contrast to other cellular RNA species, the ribosomal RNA´s occur in very high copy number per active cell (up to 16.000). This enables an efficient labeling of physiologically active cells with rRNA-targeting fluorescence labeled probes. For a first screening of bacterial isolates from any plant environment, a large set of phylogenetic oligonucleotide probes (Table 1) can be used in a hierarchical manner from the kingdom down to the genus and species level (Amann *et al*., 1995). Bacterial isolates of known species can be classified in a few hours and candidates of possibly not yet described phylogeny can be identified usually at the subphylum or genus level at least, using the fluorescence *in situ* hybridization (FISH) technique (see Section 2. 4.).

A more detailed phylogenetic analysis is necessary in the case of isolates with possibly new or uncertain phylogenetic classification by sequence analysis of the 16S-rDNA. Using PCR primers complementary to the highly conserved 5´- and 3´-ends of the 16S-rDNA-coding genes, ribosomal RNA sequences of pure isolates or of 16S-rDNA clones retrieved from complex microbial communities (see below) can be obtained. For reliable identification, the complete 16S-rDNA sequence has to be used for phylogenetic analysis. The powerful software package, ARB (Ludwig, www link), has several sequence-analysis tools, *e.g*., for tree calculation, available. Specific oligonucleotide probes can be developed by the implemented PROBE DESIGN and PROBE MATCH tools. In order to have a cultivation-independent analysis of the plant-associated bacterial diversity, the 16S-rRNA or 16S-rDNA of the RNA / DNA extracted from natural habitats needs to be PCR-amplified (in the case of RNA, after a reverse-transcriptase step) and cloned. The analysis of the 16S-rDNA clones is performed as described above, yielding insight into the natural diversity. After improving the set of probes for expected bacteria, the *in situ* analysis with FISH can be performed to close the "cyclic rRNA-approach" (Figure 1). A similar approach has been performed using the *nif* genes of natural plant-associated communities (Hurek *et al*., 2002). However, an *in situ* labeling by FISH that targets *nif*-mRNA is much less efficient because of the lower copy number of mRNA as compared to rRNA.

2.2. Additional Molecular Markers

Due to the sometimes very close phylogenetic relationships, alternative molecular markers, such as either the 23S-rDNA or the IGS-regions of the r-DNA operon,

have to be used for successful phylogenetic identification. For a final decision on the phylogenetic relationship of bacterial isolates, the DNA-DNA relatedness of the entire bacterial DNA has to be examined and a polyphasic identification approach has to be performed (Vandamme *et al*., 1996), in addition to the 16S-rDNA similarity analysis. Above the level of 70% DNA similarity, bacteria are defined to belong to the same species. Other biochemical markers, such as protein profiling, fatty-acid analysis, and DNA-fingerprinting techniques, support this type of classification and can separate the diversity of bacterial isolates at the subspecies or microdiversity level (Rademaker *et al*., 2000, Schloter *et al*., 2000).

Table 1. Phylogenetic rRNA-targeting oligonucleotide probes

Probe	Position	Sequence	% FA	Specificity	Ref
		***In situ* probing of *Azospirillum* spp.**			
AZO-440a	16S, 440-457	GTCATCATCGTCGCGTGC	50	*Azospirillum* spp. *Skermanella*, *Rhodocista*	a
AZO-440b	16S, 440-457	GTCATCATCGTCGTGTGC	50	*Azospirillum* spp. *Skermanella*, *Rhodocista*	a
AZOI-655	16S, 655-672	CACCATCCTCTCCGGAAC	50	Species cluster: *A. lipoferum*, *A. brasilense*, *A. halopraeferans*, *A. doebereinerae*	a
Aama-1250	16S, 1250-1267	CACGAGGTCGCTGCCCAC	50	*A. amazonense*	a
Abras-1420	16S, 1420-1438	CCACCTTCGGGTAAAGCCA	40	*A. brasilense*	a
Adoeb-587	16S, 587-604	ACTTCCGACTAAACAGGC	30	*A. doebereinerae*	b
Ahalo-1115	16S, 1115-1133	ATGGTGGCAACTGGCAGA	45	*A. halopraeferans*	a
Ahalo-1249	16S, 1249-1266	GCGACGTCGCTTCCCACT	60	*A. halopraeferans*	a
Airak-1423	16S, 1423-1440	CACCGGCTCAGGTAAAG	10	*A. irakense*-cluster	a
Airak-985	16S, 985-1003	TCAAGGCATGCAAGGGTT	35	*A. irakense*-cluster	a
Alila-1113	16S, 1113-1130	ATGGCAACTGACGGTAGG	35	*A. lipoferum*, *A. largimobile*	a
Ahalo-1115C	16S, 1115-1133	ATGATGGCAACTGGCAGTA	45	Competitor	a
Ahalo-1249C	16S, 1249-1266	GCGACTTCGCTTCCCACT	60	Competitor	a
Abras-1420C	16S, 1420-1437	CACCTTCGGGTAAAACCA	40	Competitor	a
Alila-1113-C	16S, 1113-1130	ATGGCAACTGGCGGTAGG	20	Competitor	a
		***In situ* probing of *Herbaspirillum* spp.**			
HERB-1432	16S, 1432-1449	CGGTTAGGCTACCCAACTT	35	Genus *Herbaspirillum*	c
Hrubri-445	16S, 445-462	GCTACCACCGTTTCTTCC	60	*H. rubrisubalbicans*	c
Hsero-445	16S, 445-462	GCCAAAACCGTTTCTTCC	35	*H. seropedicae*	c
Hfris-445	16S, 445-462	TCCAGAACCGTTTCTTCC	50	*H. frisingense*	c

***In situ* probing of *Burkholderia* spp.**

Subu-1237	16S, 1237-1254	AATGGTCGGAACAGAGGG	60	Genera *Burkholderia* and *Suturella*	d
Bcv-13b	23S, 255-274	ACAGGGCACGTTCCGATGT	25	*B. cepacia, B. vietnamiensis,* Gruppe VI, *B. multivorans, B. stabilis, B. pyrocina*	d
Bglad-445	16S, 445-463	GCCCTCAGGATTTCTTTC	35	*B. gladiolii*, *B. glumae, B. vandii*, *B. plantarii*, *B. cocovenenans*	d
Bglad-465	16S, 465-482	GTCATCCCCGAAGGATAT	35	*B. gladiolii*, *B. glumae*, *B. plantarii*, *B. cocovenenans*	d
Bbras-636	16S, 636-653	CCAGCGCTGCAGTCACCA	60	*B. brasilense*	e
Bbras-62	16S, 62-79	AGCCCGCGCTGCCGTCCG	60	*B. brasilense*	e
Btrop-636	16S, 636-653	CAAGCGATGCAGTCACCA	55	*B. tropica*	f
Btrop-463	16S, 463-480	CATCCCCCGGCCATATTA	20	*B. tropica*	f

FA = % Formamide in the hybridization buffer; *a, Stoffels* et al.*, 2001; b, Eckert* et al.*, 2001; c, Kirchhof* et *al., 2001; d, Stoffels* et al.*, 1998; e, Baldani and Hartmann, 2003; f, Reis* et al.*, 2004.*

For diversity analysis that targets the functional genes of nitrogen fixation, the rather highly conserved *nifHDK* operon is used. There have been several studies to compare the 16S-rDNA-based and either the *nifH*-gene or *nifH*-protein sequences. This is necessary to confirm that the evolutionary relationships are also applicable for this particular gene. Hennecke *et al.* (1985) investigated the relative few *nif*-gene sequences available at that time and showed that the *nifH*-sequence data support the 16S-rRNA tree; this also holds true for the *nifD* and *nifK* sequences. Normand and Bousquet (1989) performed a similar study that included *Frankia* and other Firmicutes and showed a clustering in the Gram-positive bacteria. However, lateral gene transfer, especially in closely related species, could not be excluded.

Evidence for lateral gene transfer emerged, for example, from studies of the *nifHDK* genes of the β-proteobacterium *Azoarcus* (Hurek *et al.*, 1997). Recently, different primers and probes targeting the *nifH* gene have been successfully used for phylogenetic analysis of diversity of diazotrophic bacterial communities in soil and rhizosphere environments (Ueda *et al.*, 1995; Zehr *et al.*, 1998; Widmer *et al.*, 1999; Mergel *et al.*, 2001). Interestingly, these studies demonstrated a high diversity of environmental *nifH* sequences, which clearly exceeds the sequence diversity of the hitherto known and cultivated diazotrophs. It is quite possible that *nif* genes are much more widely distributed among currently known bacteria; further, *nif* genes

could also be present in the unculturable (or not cultured) fraction of bacteria in the environment.

2.3. Diversity Studies using Molecular Probing and Fingerprinting Techniques

In addition to 16S- and 23S-rRNA oligonucleotide probes, a number of other molecular taxonomic tools, *e.g*., RAPD-markers (Vaneechoutte, 1996), RFLP-analysis (Han and New, 1998), and rep-PCR analysis (Rademaker *et al*., 2000), have been developed and successfully applied. These approaches allow the rapid molecular identification at the species and even subspecies down the individual strain level. The RFLP-analysis of the whole genome with rarely cutting restriction enzymes, followed by pulsed field-gel electrophoresis, can be used for strain specific identification (Gündisch *et al*., 1993). These and related studies have also shown that the 16S-rDNA genes of many bacteria do occur in multiple replicons (Gündisch *et al*., 1993; Caballero-Mellado *et al*., 1999).

2.4. Molecular Tools for in situ *Localization and Population Dynamic Studies*

Fluorescence *in situ* hybridization (FISH), using fluorescence-labeled oligonucleotide probes, provides a handy molecular tool not only to screen and characterize the phylogeny of bacterial isolates, but even more to get insight into the localization of individual cells in their natural habitat without the need of cultivation (Amann *et al.*, 1995, Wagner *et al.*, 2003). After a fixation step of the material, usually in paraformaldehyde (3%) overnight at 4^{o}C, the material is fixed on glass slides and dehydrated with an increasing ethanol series. According to Wagner *et al.* (1993), the FISH protocol uses an incubation at 46°C for 90 min in an hybridization buffer containing 0.9 M NaCl and different concentrations of formamide according to the stringency conditions for the probes used (see Table 1). This step is followed by a stringent washing step at 48^{o}C for 15 min in a buffer with optimized NaCl concentrations and EDTA. After rinsing the slides with water, the samples can also be counterstained with DAPI as a general DNA-stain and mounted in anti-fading solution. Regular epifluorescence microscopy is usually not sufficient for environmental and root samples because of the autofluorescence problem of the biological matrix. Confocal laser scanning microscopy, equipped with two lasers (Ar and HeNe, supplying excitation wave length at 365, 488, 543 and 633 nm) provides much better resolution (as reviewed by Hartmann *et al.*, 1998).

An alternative very powerful fluorescence-based *in situ* detection method is the fluorescence tagging of bacteria with either the *gfp*- (green fluorescence protein) or *rfp*- (red fluorescence protein) gene (Unge *et al.*, 1998). This molecular tagging is used either for general cell tagging or in operon fusion constructs for expression studies of genes of interest, *e.g*., the *nifH* gene (Egener *et al.*, 1998).

If population studies do not focus on a high spatial resolution, *ex situ* molecular analyses of the bacterial diversity can be performed as summarized by Hartmann *et al.* (2003). Genomic DNA and rRNA are isolated from the biological material following standard protocols (Miethling *et al.*, 2000). Usually, a further purification of the RNA/DNA extract is necessary. For amplification of the desired specific

DNA range, either a primer specific for 16S-rDNA coding genes, *e.g.*, 616-F and 630-R (Juretschko *et al.*, 1998) or specific *nifH*-targeting primers (Ueda *et al.*, 1995, Widmer *et al.*, 1999) are used. The resolution of the diversity of PCR-amplificates can be resolved by temperature-gradient gel electrophoresis (TGGE), as described by Heuer and Smalla (1997), to separate high molecular diversity DNA species according to their sequence. The separated DNA-amplificates are finally analyzed by sequencing to retrieve information about their affiliation and underlying diversity. If very high resolving molecular diversity analysis is needed, the method of choice is the development of clone libraries of long amplification products, which provides the basis for most comprehensive phylogenetic diversity analyses.

3. MOLECULAR PHYLOGENY AND ECOLOGY OF *AZOSPIRILLUM* AND OTHER NITROGEN-FIXING α-SUBCLASS PROTEOBACTERIA

3.1. Diversity of Diazotrophic α-Proteobacteria

The nitrogen-fixing bacteria of the α–subclass of Proteobacteria mostly occur in six major groups. One cluster contains *Zymomonas*, *Rhizomonas*, and *Sphingomonas*, plus a diazotrophic bacterium isolated from rice and characterized as *Sphingomonas paucimobilis.* A second group harbors the endophytic diazotroph, *Gluconacetobacter* spp., now with three species, *G. diazotrophicus* from sugar cane (Gillis *et al*., 1989), and *G. johannae* and *G. azotocaptans* from coffee plants (Fuentes-Ramírez *et al*., 2001). The former species, *Acetobacter diazotrophicus*, (Gillis *et al.*, 1989) was reclassified as *Gluconacetobacter diazotrophicus* based on comparative sequence analysis of 16S-rRNA sequences (Yamada *et al.*, 1997). A third small group comprises the genera *Rhodobacter*, with the diazotrophic *Rhodobacter capsulatus*, and *Paracoccus*. A fourth large cluster represents the symbiotic genera, *Rhizobium*, *Sinorhizobium, Mesorhizobium,* and *Ochrobactrum*. Very recently, a nitrogen-fixing *Ochrobactrum* sp. was isolated from *Acacia* nodules; it belongs to the Rhizobiaceae family and forms fully developed and functional nodules with roots of *Acacia* (A. Ngom, personal communication). The fifth cluster is formed by the symbiotic bacteria, *Bradyrhizobium* and *Azorhizobium*, as well as *Beijerinckia*, *Xanthobacter*, and *Rhodopseudomonas*. The last group comprises the nitrogen-fixing genera, *Azospirillum*, *Rhodospirillum*, *Aquaspirillum*, and *Magnetospirillum* in the so-called α-1-subclass.

3.2. Diversity of Azospirillum spp.

In the rediscovery of *Azospirillum* in the 1970's by Dr. Johanna Döbereiner and her associates (Tarrand *et al*., 1978), the species *A. lipoferum* and *A. brasilense* were described. They resembled *Spirillum lipoferum* originally described by Beijerinck in 1925. In the following years, *A. amazonense* (Magalhães *et al*., 1983)*, A. irakense* (Khammas *et al*., 1989), *A. halopraeferens* (Reinhold *et al.,* 1987), and *A. largimobile* (Sly and Stackebrandt, 1999) were discovered (see Okon, 1994). The most recently identified *Azospirillum* species is *A. doebereinerae*, which was

isolated from roots of the giant C4-grass *Miscanthus sinensis* (Eckert *et al*., 2001). The taxonomy, physiology and ecology of the genus *Azospirillum* were recently reviewed by Baldani and Hartmann (2003).

3.2.1. 16S-rDNA-based Molecular Phylogeny

In a detailed 16S-rDNA-based molecular phylogenetic study, Stoffels *et al*. (2001) demonstrated that the presently known seven species of *Azospirillum* form a phylogenetic cluster together with *Skermanella* and *Rhodocista. A. brasilense*, *A. lipoferum, A. doebereinerae, A. largimobile* and *A. halopraeferens* constitute one subcluster, whereas *A. irakense, A. amazonense* and *Rhodocista* form a second, and *Skermanella* forms a third subcluster (Figure 2). The DNA G+C content for these species is in the range of 64-71%. The 16S-rDNA-sequence similarity between the different species is in the range of 93.6-96.6% within one subcluster and 90.2-90.6% between the species members of two subclusters. Accordingly, on the basis of different more or less conserved sequence stretches of the 16S-rDNA, it was possible to create a set of oligonucleotide probes with different degrees of specificity from the whole cluster (probe AZO-440a+b), to a subcluster (AZOI-665), and to individual-species levels, *e.g*., probes Abras-1420, Alila-1113, Adoeb-587, Ahalo-1249, Aama-1250 and Airak-1423 (Stoffels *et al*., 2001; see Table 1). To block specifically cross-reacting 16S-rRNA species, unlabeled oligonucleotide probes as competitors were suggested (Stoffels *et al*., 2001).

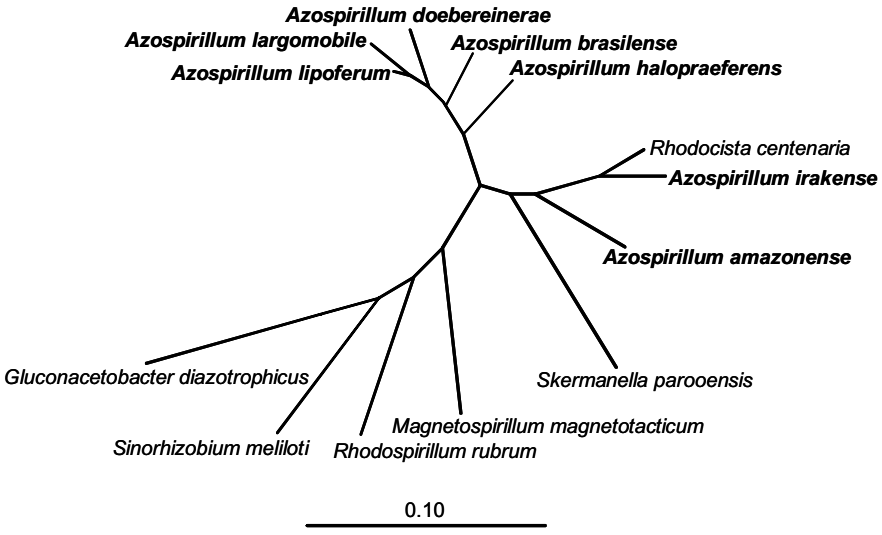


Figure 2. 16S-rDNA phylogenetic tree of Azospirillum *spp. and related α-Proteobacteria*

3.2.2. Use of Phylogenetic Probes for in situ *Localization*

Oligonucleotide probes with fluorescence labels, like FLUOS, TRITC, Cy3 or Cy5, are applied in fluorescence *in situ* hybridization (FISH) of fixed bacterial cells and root samples for identification and *in situ* localization purposes. Because these probes were designed for different hierarchical levels, their application in a nested approach allows very reliable identification (Figure 3). The application of confocal laser scanning microscopy (CLSM) is necessary to reduce the out-of-focus fluorescence in root samples. Using this approach, single bacterial cells of *A. brasilense* Sp7 were identified and localized preferentially at the root surface, whereas the strain *A. brasilense* Sp245 was also found endophytically in the intercellular spaces of the root epidermis. Alternatively, a specific *in situ* monitoring of introduced bacteria could be performed using *gfp*- or *rfp*-labeled bacterial strains (Rothballer *et al*., 2003).

3.3. Diversity and Ecology of Gluconacetobacter *spp.*

Specific PCR-primers have been developed to identify *G. diazotrophicus* (Kirchhof *et al*., 1998) and *G. johannae* as well as *G. azotocaptans* (Fuentes-Ramírez *et al*., 2001). Using the primers L927Gj and L923Ga, *G. johannae*- and *G. azotocaptans*-specific amplification of a 400-bp fragment can be used for both specific identification and semi-quantitative estimation of the occurrence of these important endophytic diazotrophs.

G. diazotrophicus has now been isolated from coffee (Jiménez-Salgado *et al*., 1997) and pineapple plants (Tapia-Hernández *et al.,* 2000) as well as from sugarcane. Its infection of sugarcane has been investigated with electron-microscopy techniques (James *et al*., 1994; 2001) and reviewed (James and Olivares, 1997). The benefit of *G. diazotrophicus* inoculation to sugarcane was investigated (Sevilla *et al*., 2001) by using both wild type and Nif^- mutant strains, and demonstrated both an effect of nitrogen fixation and a likely phytohormone-mediated growth stimulation (see Chapter 10, this Volume).

4. MOLECULAR PHYLOGENY AND ECOLOGY OF *HERBASPIRILLUM,* DIAZOTROPHIC *BURKHOLDERIA* SPP., AND OTHER N_2-FIXING β-PROTEOBACTERIA

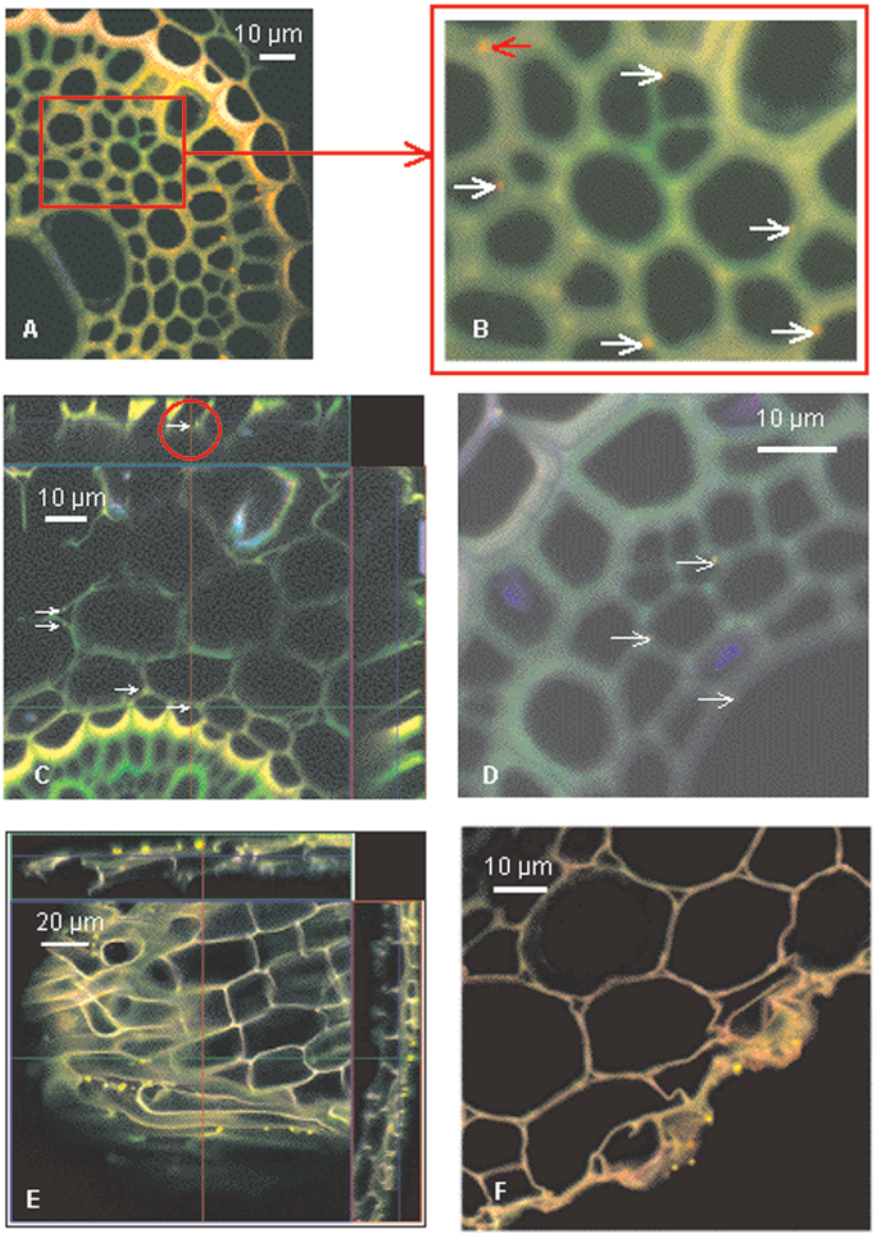
4.1. Diversity of Diazotrophic β-Proteobacteria

Among the β-Proteobacteria, the number of known diazotrophic bacteria has increased much in the last decade. Young (1992) reported four diazotrophic genera in the β-Proteobacteria; *Alcaligenes*, *Rhodocyclus, Derxia,* and *Thiobacillus*. In the meantime, *Alcaligenes paradoxus* was grouped with the genus *Variovorax* and *Rodocyclus gelatinosus* with *Rubrivivax.* Nitrogen-fixing isolates of *Ideonella dechloratans* were obtained from rice (Elbeltagy *et al*., 2001). Other newly described bacteria that originate from different plants include *Azoarcus* and related new diazotrophic genera (Reinhold-Hurek and Hurek, 1998; 2000; see Chapter 9),

several new *Herbaspirillum* species, and an increasing number of diazotrophic *Burkholderia* species. Until recently, true nodule-forming bacteria on *Leguminosae* were only known within the *Rhizobiaceae* (α-Proteobacteria). Chen *et al*. (2001) have now isolated bacteria from root nodules on *Mimosa pudica* and *Mimosa diplotricha*, and from cystic-fibrosis sputum, that was described as a novel *Ralstonia* species, *Ralstonia taiwanensis*.

The *R. taiwanensis* isolates from *Mimosa* nodules were proved to effectively nodulate the *Mimosa* species and were the first described β-proteobacteria capable of both nitrogen fixation and root-nodule formation. In the same year, Moulin *et al.* (2001) reported two *Burkholderia* strains STM678 and STM815, which represent the new species *B. phymatum* and *B. tuberum* (Vandamme *et al.*, 2002) that were isolated from two tropical legume plants, *Aspalathus carnosa* in South Africa and *Machaerium lunatum* in French Guiana. Nodulation was demonstrated by these cultures, which harbor nodulation genes that resemble the *nod* genes in α-Proteobacteria. In addition, two other *Burkholderia* isolates, belonging to *B. caribensis* and *B. cepacia* genomovar VI, were obtained from root nodules of *Mimosa* spp. in Taiwan and *Alysicarpus glumaceus* in Senegal, respectively (Vandamme *et al*., 2002). Thus, the concept of "β-Rhizobia" arose, with some β-Proteobacteria being capable of nodulating legumes. The genetic capacity for this symbiotic trait may have spread, *e.g.*, by plasmid transfer, to root-associated diazotrophs.

Figure 3 (opposite). In situ *localization of associated/endophytic nitrogen fixing bacteria.*
***A**: CLSM image of a radial slice of a barley root (magnification 400x, scan zoom 1,5). Image shows endophytic colonization of the central cylinder with* Burkholderia cepacia *SXO702.*
***B**: Magnification shows bacteria in the intercellular space (apoplast). Some bacteria are attached to the inner surface of the cell wall.*
***C:** Orthogonal view of a radial slice of barley roots inoculated with* Herbaspirillum seropedicae *Z67 (magnification 400 x, Scan zoom 1,5). Cells were found in the root cortex (white arrows).*
***D:** Confocal image of a radial slice of barley roots (magnification 400 x, Scan zoom 2,0).* Herbaspirillum seropedicae *Z67 shows endophytic colonization of the central cylinder.*
***E:** Colonization of wheat roots (Cultivar Naxos) harvested after two weeks of cultivation in a monoxenic system inoculated with* Azospirillum brasilense *Sp7(magnification 630 x, Scan zoom 1,4).*
***F:** Radial slice of a barley root inoculated with* Azospirillum brasilense *Sp7 under the same culture conditions as in E (magnification 400 x, Scan zoom 1,8).*
***A - D, F:** Bar indicates 10 µm,* ***E:** Bar indicates 20 µm.*
A, B: In situ *hybridization was performed with the oligonucleotide probes Bcv-13b-Cy3 and EUB-338-I, II, III-FLUOS (Table 1).*
C, D: In situ *hybridization was performed with the oligonucleotide probes Hsero-445-Cy3 and EUB-338-I, II, III-FLUOS (Table 1).*
E, F: In situ *hybridization was performed with the oligonucleotide probes Abras-1420-Cy3, EUB-338-I, II, III- FLUOS (Table 1).*
Bacterial cells appear in orange/yellow after in situ *hybridization according to the overlay of the Cy3 (red) and FLUOS (green) signal.*


10 µm
A
B
10 µm
C
10 µm
D
20 µm
E
10 µm
F

A comparable observation was made among in rhizobial populations in the field (Sullivan *et al.*, 1995). A symbiotic island was transferred to inefficient nodulating *Rhizobium loti* making them very efficient symbiotic strains. Many more nitrogen-fixing and nodulating β-proteobacteria may exist, but remain to be described because they have not yet been tested for their nodulation ability. Plant endophytic diazotrophs, which were found over the recent years in several genera of β-Proteobacteria, *e.g.*, *Herbaspirillum* and *Burkholderia,* could be ideal candidates for these new types of symbiotic bacteria.

4.2. Diversity and Ecology of Herbaspirillum spp.

The first described species of the genus *Herbaspirillum* was *H. seropedicae* (Baldani *et al*., 1986), which included bacterial strains isolated from roots of several cereals. *Pseudomonas rubrisubalbicans*, a mild pathogen in some sugarcane varieties, was reclassified as *Herbaspirillum rubrisubalbicans* by Baldani *et al*. (1996). A third species, mostly harbouring strains from clinical origins and provisionally named *Herbaspirillum* species 3, had also to be included in the *Herbaspirillum* species because of its phylogenetic and biochemical close relatedness, although most of isolates do not fix nitrogen and are not derived from plant origin.

H. frisingense was recently isolated from roots and stems of C4-fibre plants (*Pennisetum purpureum* in Brazil and *Miscanthus sinensis* in Germany; Kirchhof *et al*., 2001). Most recently, two more species of *Herbaspirillum* were suggested. Several isolates were obtained from the nodules of *Phaseolus vulgaris* plants from Portugal and showed close relatedness to *Herbaspirillum* (Valverde *et al*., 2003). The isolates showed 92-98% DNA-DNA relatedness among each other but only 29% DNA-relatedness to the other described species. Therefore, the new species *H. lusitanum* was suggested with the type strain P6-12^{T} (Valverde *et al.*, 2003). There is also 16S-rDNA-sequence information about a fifth *Herbaspirillum* sp., named *H. chlorophenolicum*, which is able to degrade chlorophenol and was initially named *Commamonas testosterone* (Im *et al.,* 2004). The 16S-rDNA-phylogenetic tree, based on maximum likelihood analysis, is shown in Figure 4. All five *Herbaspirillum* species form a monophyletic cluster and have *Janthinobacterium lividum, Telluria mixta,* and *Duganella zoogloeoides* as closest relatives (Schmid *et al.*, 2005).

With the exception of *H. chlorophenolicum* and *Herbaspirillum* species 3, all *Herbaspirillum* spp. are nitrogen-fixers and colonize plant roots. Some *Herbaspirillum* isolates can form an endophytic association with plant tissue as seen with *Sorghum bicolor* (James *et al*., 1997) and sugarcane (Olivares *et al*., 1997). Recently, direct evidence for a nitrogen-fixing endophytic association has been obtained from the studies of *Herbaspirillum* sp. strain B501 in rice, *Oryza officinalis* (Elbeltagy *et al*., 2001).

Based on the 16S-rDNA-sequences, a set of oligonucleotide probes, which allow the identification and differentiation of *H. seropedicae, H. rubrisubalbicans*, and *H. frisingense* by species-specific probes and FISH, was suggested (Kirchhof *et al*., 2001). Using these probes for the screening of new isolates from different sources,

evidence for the presence of *Herbaspirillum* spp. was found in many other plants, including pineapple, banana, and rice (Weber *et al*., 1999; Cruz *et al*., 2001). Both 16S-rRNA-targeted oligonucleotide probes (Table 1) and FISH-analysis were used to localize these bacteria in the root environment. Figure 3 shows the *in situ* localization of an isolate of the proposed species *H. lusitanum* from barley roots colonizing the root endophytically.

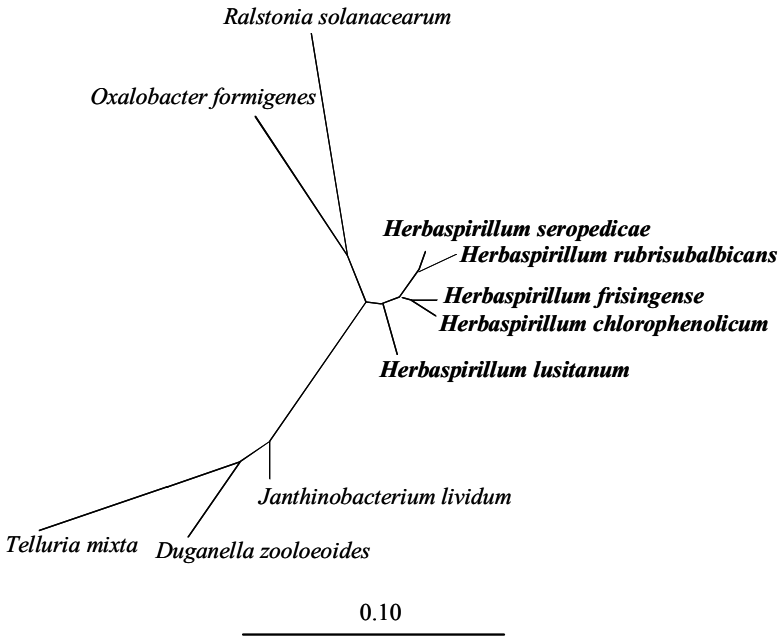


Figure 4. 16S-rDNA phylogenetic tree of Herbaspirillum *spp.*

An endophytic location for *H. seropedicae* has been repeatedly described in roots, shoots and leaves of Gramineae (Olivares *et al.*, 1996) as has its colonization behaviour and systemic spreading in the vascular tissue of *Sorghum bicolor* (James *et al.*, 1997). Similarly, in an axenic system, *H. frisingense* efficiently and endophytically colonized and systemically spread through micro-propagated *Miscanthus* seedlings (Eckert, unpublished results).

4.3. Diversity and Ecology of Diazotrophic Burkholderia *spp.*

An unprecedented high diversity of diazotrophic root-associated bacteria has been found in recent years within the genus *Burkholderia*. Among the presently known 29 different *Burkholderia* species or genomovars of the genus *Burkholderia* are

many plant- and human-associated bacteria with high pathogenic potential, especially in the *B. cepacia* cluster. In addition, some *Burkholderia* are degraders of organic substances of anthropogenic origin and others are plant-growth-promoting bacteria, some with biocontrol activity. The first diazotrophic species within the genus *Burkholderia* was *B. vietnamiensis* (Gillis *et al*., 1995). This species was isolated from the rhizosphere of young rice plants grown in Vietnamese soil (Tran *et al*., 1994). It also includes two clinical isolates, which are able to fix nitrogen and were misnamed as *Pseudomonas cepacia* (Yabuuchi *et al*., 1992; Palleroni, 1993).

In a survey of root-associated diazotrophs in sugarcane and rice in Brazil, a group of diazotrophic isolates were obtained using the LGIP semisolid nitrogen-free medium, usually applied to isolate *G. diazotrophicus* (Reis *et al*., 1994); these were provisionally named "isolates E" (Oliveira, 1992). Application of phylogenetic oligonucleotide probes characterized these bacteria as β-Proteobacteria of probably new phylogeny. Concomitant sequence analysis of 23S-rDNA-coding genes indicated the affiliation of these bacteria to the genus *Burkholderia* (Hartmann *et al*., 1995). In addition, among diazotrophic bacterial isolates obtained from banana and pineapple rhizospheres in Brazil, several isolates were found to belong with these new diazotrophic bacteria, using 23S-rRNA-oligonucleotide probing, ARDRA-pattern analysis, and phenotypic techniques (Weber *et al*., 1999; Magalhaes-Cruz *et al*., 2001). Furthermore, among N_2-fixing bacteria associated with maize and coffee plants grown in different climatic regions in Mexico, a richness of *Burkholderia* species was characterized (Estrada-de los Santos *et al*., 2001). These observations finally led to the suggestion of a new bacterial species, *B. tropica* (Reis *et al*., 2004), which is closely related to *B. unamae*, another new diazotrophic bacterial species (Caballero-Mellado *et al*., personal communication).

The "bacteria E"-isolates from rice plants turned out to be separate *Burkholderia* species, for which the name *B. brasilensis* is suggested (Baldani *et al*., unpublished data). Surprisingly, this bacterium is very closely related to a diazotroph, which was isolated from trichloroethylene-polluted groundwater, and named *B. kururiensis* (Zhang *et al*., 2000). Most interestingly, one of the β-rhizobia, *B. tuberum* strain STM678 (Moulin *et al*., 2001; Vandamme *et al*., 2002) clusters with this group of mostly root-associated endophytes (Figure 5). The other β-rhizobial species of *Burkholderia, B. phymatum*, is closely related to *B. caribiensis* (Figure 5).

For some of these new diazotrophic *Burkholderia* spp. 16S-rRNA-targeted oligonucleotide probes are available (Table 1) and FISH analysis and confocal laser scanning microscopy could be used to study the ecology of these bacteria. Possibly, an endophytic localization might be found for some of these isolates, like *B. cepacia* SXO (Figure 3).

A *Burkholderia* sp. has also been found in association with the arbuscular mycorrhizal fungus, *Gigaspora margarita*, as a non-culturable endosymbiont (Minerdi *et al*., 2001). Molecular techniques show that this bacterium harbours the *nifH* gene. In the association with plant roots and fungi, probably many more diazotrophic bacteria (either culturable or non-culturable) are waiting to be discovered and they may surprise the scientific world with new symbiotic characters.

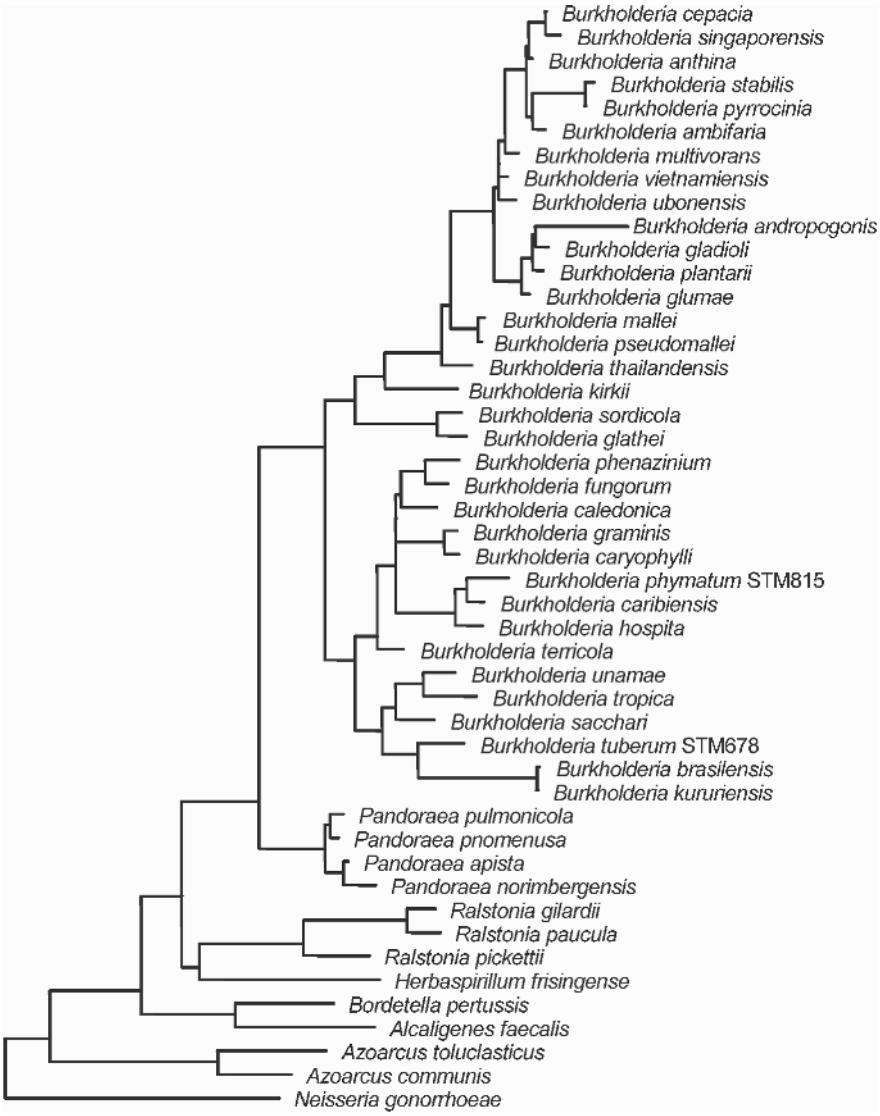


Figure 5. 16S-rDNA phylogenetic tree of Burkholderia *spp. and related β-Proteobacteria*

5. CONCLUSIONS AND PROSPECTS FOR FUTURE STUDIES

Using 16S-rRNA-directed phylogenetic oligonucleotide probes, both the phylogenetic characterization of isolates and the *in situ* identification and

localization of these bacteria in root and rhizosphere sample is possible. Due to the high fluorescence background of natural samples, the application of either confocal laser scanning microscopy or other image analysis-supported microscopic techniques using, *e.g*., the convolution method, are necessary. Because new diazotrophic bacterial species are continually being described and originating from different plants, a high diversity of hitherto unknown diazotrophs can still be expected. Therefore, the application of culture-independent approaches, using primers for the 16S-rDNA and *nif* genes, are highly recommended in future diversity studies to get an even closer insight into the real diversity of plant-associated diazotrophs. Some of these diazotrophs may have acquired a very intimate state of coevolution towards a symbiotic life style with plants and even with fungi.

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Chapter 3

REGULATION OF NITROGEN FIXATION AND AMMONIUM ASSIMILATION IN ASSOCIATIVE AND ENDOPHYTIC NITROGEN FIXING BACTERIA

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1. INTRODUCTION

A molybdenum-containing enzyme complex, termed Mo-nitrogenase, catalyzes the reduction of molecular nitrogen to ammonia. The genes required for the synthesis and function of Mo-nitrogenase were first characterized in *Klebsiella pneumoniae* M5a1, a strain closely related to *Klebsiella* strains isolated from plants. Twenty genes that are organized in 8 transcription units have been characterized in this strain and most of them are conserved and are common to all nitrogen fixers studied so far (Arnold *et al.,* 1988; Elmerich, 1991; Merrick, 1992; 1993). The Mo-nitrogenase polypeptides are encoded by three structural genes, *nifDK* for nitrogenase component 1 (or MoFe protein) and *nifH* for nitrogenase component 2 (or Fe protein). Full assembly of nitrogenase requires the products of other *nif* genes that are involved either in the processing of nitrogenase metalloclusters and catalytic stability (*nifMZ, nifW, nifUS*) or in the synthesis of a specific molybdenum cofactor bound to component I (*nifBQ, nifENX, nifV* and *nifH*) (Dean and Jacobson, 1992; Rangaraj and Ludden, 2002).

Regulation of *nif*-gene expression is complex and it is coupled to the regulation of ammonia assimilation and nitrogen metabolism through the nitrogen regulatory system (Merrick and Edwards, 1995). The *ntr* system is a transcriptional/post-translational regulatory cascade for both the expression of genes and for the control of activity of gene products of nitrogen metabolism in response to ammonium

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concentration. It involves the two-component response regulators, *ntrBC/YX*, and *glnB*-like genes. The PII-protein family contains signal-transduction trimeric proteins that are central to the regulation of nitrogen metabolism in prokaryotes. The biochemistry and physiology of the PII signal-transduction proteins has been reviewed recently (Arcondéguy *et al.,* 2001). Expression of *nif* genes in Proteobacteria depends of a specific transcription regulator, called NifA, together with a particular sigma factor, σ^{54} (also designated σ^{N}), which is encoded by *rpoN* (Dixon, 1984; 1998; Barrios *et al.,* 1999).

This chapter deals with the structural organization and regulation of genes that are involved in nitrogen fixation, ammonium transport and assimilation, and with the general nitrogen regulatory system in associative/endophytic bacteria belonging to the genera *Azoarcus, Azospirillum*, *Gluconacetobacter, Herbaspirillum*, and *Pseudomonas*. It also deals with interactions of gene products that define signal transduction in the control of metabolic responses in these systems. For reviews on early microbiological, ecological, taxonomical, physiological, biochemical, and genetic studies with these organisms, see Döbereiner and Pedrosa (1987), Elmerich *et al*. (1987; 1992; 1997), Okon (1994), Baldani *et al.* (1997), Pedrosa *et al.* (1997), Rudnick *et al.* (1997), and Sevilla *et al.* (1997). Among Proteobacteria of the γ–subgroup, representatives of *Klebsiella* and *Azotobacter* species are commonly isolated from the rhizosphere (Döbereiner and Pedrosa 1987). The *nif* genetics of both *K. pneumoniae* and *Azotobacter vinelandii* is detailed in *Genetics and Regulation of Nitrogen Fixation in Free-living Bacteria*, volume 2 of this series. The genetics and physiology of free-living and plant-associated cyanobacteria is covered in Chapters 11 and 12 of this volume.

2. RHIZOSPHERIC AND ENDOPHYTIC BACTERIA: GENERAL FEATURES

Azospirillum species are Gram-negative vibrioid diazotrophs that are found associated with several plants of agronomic importance, such as wheat, rice, sorghum and maize, and with several non-graminaceous species; they occur on root surfaces and, in some cases, in the first layers of the cortex (Baldani *et al.,* 1997). The *Azospirillum* genus was described by Tarrand *et al*. (1978) and initially contained the species, *A. brasilense* and *A. lipoferum*. Presently, the genus *Azospirillum* comprises seven recognized species: *A. brasilense, A. lipoferum, A. amazonense, A. irakense*, *A. halopraeferens*, *A. largimobile*, and *A. doebereinerae* (see references in Chapter 2). Bacteria of the genus *Azospirillum* belong to the α–Proteobacteria (Young, 1992).

The initial studies of the genetics, molecular biology, physiology, and biochemistry of nitrogen fixation in *A. brasilense* and *A. lipoferum* were reviewed by Döbereiner and Pedrosa (1987) and Elmerich *et al*. (1992). These reviews describe the isolation of early *nif*, *gln* and *ntr* mutants, metabolite-resistant mutants, chemical and insertional mutagenesis, and genetic elements. They also describe the early evidence for chromosomes or megaplasmids, bacteriophages and plasmid constitution, gene mobilization by conjugation and transformation, the non-plasmidial localization of *nif* genes, and early studies of the regulation of nitrogen fixation. The literature pertaining to the regulation of *nif*-gene expression and

nitrogen metabolism in *Azospirillum*, which focussed on the control of NifA synthesis and activity, the role of NtrBC and RpoN (σ^{54}) in nitrogen metabolism, and the involvement of PII in controlling NifA activity was reviewed by Elmerich *et al*. (1997). Additional information on *Azospirillum* carbon and energetic metabolism was reviewed by Hartmann and Zimmer (1994; see also Chapter 5).

The genome structures of five species of *Azospirillum* (namely *A. brasilense*, *A. lipoferum, A. irakense, A. amazonense* and *A. halopraeferens*) are complex and display multiple replicons, circular and linear chromosomes, and plasmids (Caballero-Mellado *et al.,* 1999; Martin-Didonet *et al.,* 2000). The nitrogenase structural genes are located in the largest replicons of *Azospirillum* species, including *A. brasilense* strains Sp7 and FP2 (Martin-Didonet *et al.,* 2000).

The genus *Herbaspirillum* (Baldani *et al*., 1986) comprises *H. seropedicae*, *H. rubrisubalbicans* (Baldani *et al.*, 1996), and *H. frisingense* (Kirchhof *et al.,* 2001). These species belong to the β–Proteobacteria (Young, 1992). *Herbaspirillum* species are Gram-negative vibrioid endophytic nitrogen-fixing bacteria that are found associated with rice, maize, sorghum, sugarcane, banana, and pineapple (see Chapter 10 of this volume). A fourth species, still unnamed, which contains clinical isolates, was also proposed (Baldani *et al.,* 1996). The regulation of nitrogen fixation by *H. seropedicae* has been reviewed recently (Souza *et al.,* 2000b; Pedrosa *et al.,* 2001).

The genus *Azoarcus* (Reinhold-Hurek *et al*., 1993) has two endophytic nitrogen-fixing species, *A. indigens* and *A. communis*, which were isolated from the roots of the salt-tolerant kallar grass, *Leptochloa fusca* (L.) Kunth. This genus also contains several non-diazotrophic, but denitrifying and aromatic compound-degrading, species. As for *Herbaspirillum*, the *Azoarcus* species belong to the β–Proteobacteria (Reinhold-Hurek *et al.,* 1993). The best characterized endophytic diazotrophic strain is the unclassified strain, *Azoarcus* sp. BH72, which clusters as "authentic" *Azoarcus* in a monophyletic unit with those of the γ–Proteobacteria, in accordance with 16S-ribosomal DNA and *nifH* phylogeny (Hurek *et al*., 1997; Egener *et al.,* 2002). The presence of *nifL* and the absence of the conserved Cys motif in the central C-terminal domain of the NifA protein strongly point to strain BH72 belonging to the γ–Proteobacteria subclass. Work on *A. indigens* and *A. communis* could shed light on this controversy, because both species cluster with strain BH72.

Cavalcante and Döbereiner (1988) reported a new acid-tolerant nitrogen-fixing bacterium that was associated with sugarcane. A collection of isolates, similar to strain Pal5, were initially classified as *Acetobacter diazotrophicus*, but then later transferred to a new genus, *Gluconacetobacter*, as the species *G. diazotrophicus* (Yamada *et al.,* 1997). Two new species of *Gluconacetobacter*, G. *johannae* and *G. azotocaptans*, were isolated from coffee plants (Fuentes-Ramírez *et al.,* 2001). The best studied species is *G. diazotrophicus*. As Azospirilla, the *Gluconacetobacter* group belongs to the α-Proteobacteria subclass. *G. diazotrophicus* and *H. seropedicae* are considered to be endophytes and have been reported to contribute significant fixed-nitrogen to sugarcane (see Chapter 10).

Early reports of nitrogen fixation by *Pseudomonas* were reviewed by Döbereiner and Pedrosa (1987) and Young (1992). Later, Chan *et al.* (1994) pointed out that several strains, originally described as putative nitrogen-fixing "*Pseudomonas*", have been reassigned to genera in the β–subgroup of Proteobacteria. This is indeed the case for *Pseudomonas saccharophila* (Barraquio *et al*., 1986), *Pseudomonas pseudoflava* (Jenni *et al.,* 1989; now re-named *Hydrogenophaga pseudoflava*), and *Pseudomonas rubrisubalbicans* (now *Herbaspirillum rubrisubalbicans*; Baldani, *et al.,* 1996), belonging to Burkholderiales. Several genuine *Pseudomonas* that belong to *Pseudomonas stutzeri* were described as nitrogen fixers (Krotzky and Werner, 1987; Vermeiren *et al*., 1999). The best characterized *Pseudomonas* for nitrogen fixation is *P. stutzeri* strain A1501, formerly *Alcaligenes faecalis* A15 (Vermeiren *et al*., 1999; Desnoues *et al*., 2003). It belongs to the γ–Proteobacteria as do *K. pneumoniae*, *A. vinelandii, Pantoea agglomerans* (formerly *Enterobacter agglomerans*), and *Enterobacter cloacae*.

3. STRUCTURAL ORGANIZATION OF *NIF* GENES

In contrast to *Klebsiella pneumoniae*, where the *nif* genes cluster in a DNA fragment of about 24 kb (Arnold *et al.,* 1988), the *nif* genes of α– and β–Proteobacteria, as well as those from the γ–subgroup, such as *Azotobacter* and *P. stutzeri*, are usually scattered in functional units throughout the genome. They are usually interspersed with electron transport protein-coding genes, such as ferredoxins (*fdx*), Mo-transport *mod* genes, chemotaxis *mcp* genes, and *fix* genes (Figure 1). Common occurrences in these clusters are genes involved in Mo metabolism and a large number of genes for probable proteins of unidentified functions (*orf's*) (Merrick, 1993).

3.1. Nitrogen-Fixation Genes in Azospirillum

A. brasilense strain Sp7 was the first of the five organisms described in this review to have its *nif* genes functionally identified and sequenced (see Elmerich *et al.,* 1997; Potrich *et al.,* 2001a). The first gene bank of *A. brasilense* strain Sp7 DNA was constructed in bacteriophage lambda (Quiviger *et al.*, 1982). A recombinant phage that carried a 6.7-kb *Eco*RI fragment, termed AbRI, was isolated by hybridization with a *K. pneumoniae nifHDK* probe. Other genes involved in nitrogen fixation, such as *nifE, nifUS* and *fixABC,* were identified in the 20-kb DNA region adjacent to *nifHDK* by Tn5 mutagenesis and hybridization with heterologous probes (Galimand *et al.,* 1989). To date, most of the genes involved in nitrogen fixation have been located in a DNA region that spans 40 kb (Figure 1) and contains 3 groups of genes organized in operons. These are: (i) *nifHDK orf1 nifY*; (ii) *nifENX orf3 orf5 fdxA nifQ, orf2 nifUSV orf4*; and (iii) *nifW* and *fixABCX* (see Elmerich *et al.,* 1997 for a review; Frazzon and Schrank, 1998; Potrich *et al*., 2001a). A region containing *nifA* and *nifB* genes (Liang *et al.,* 1991; Knopik *et al.,* 1991) was located elsewhere in the genome. The *nif*-gene organization in *A. amazonense* is likely to be similar to that of *A. brasilense* (Potrich *et al*., 2001b).

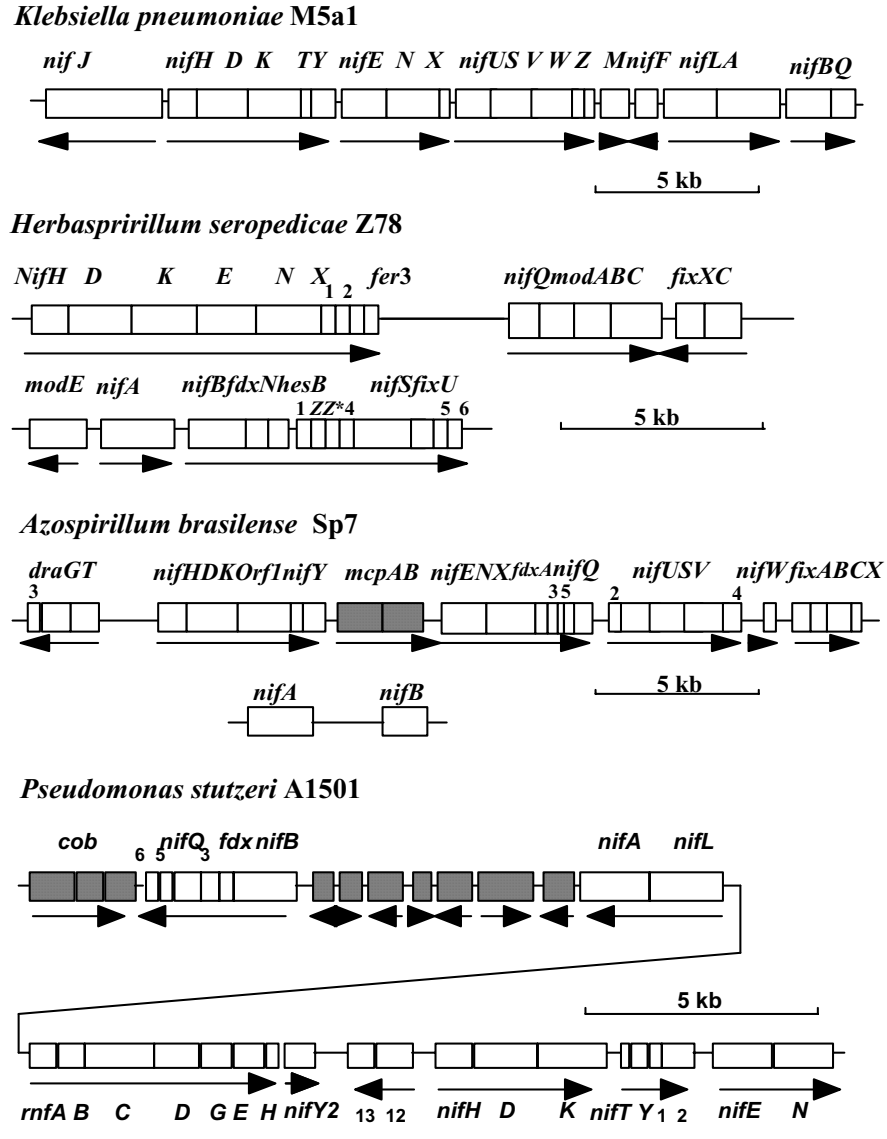

Figure 1. Organization of nif *and associated genes in associative/endophytic bacteria* A. brasilense, H. seropedicae *and* P. stutzeri. *The* K. pneumoniae nif *cluster is included for comparison. The* nif*-gene cluster of* G. diazotrophicus *is shown in Chapter 10. Horizontal arrows indicate the direction of transcription; filled rectangles indicate genes not related to the nitrogen-fixation process; and numbers indicates unidentified orfs. See text for references.*

The *draTdraG* operon, whose gene products are involved in the control of nitrogenase activity (as detailed in section 6.1), was found upstream from the *nifH* operon but transcribed divergently (for a review, see Zhang *et al.,* 1997). The same genes were found in *A. lipoferum* (Fu *et al.,* 1990). A third ORF is found downstream of *draTG* in both *A. lipoferum* and *A. brasilense* (Inoue *et al*, 1996; Ma and Li, 1997; Zhang et al. 1997).

As a recurring theme in all Proteobacteria *nif* genes, with the exception of *nifL* and *nifA* (as detailed below), their promoters contain upstream activating sequences that are specific for NifA binding (consensus: TGT N10 ACA, located about 100-150 bp upstream), and also a recognition site for σ^{54} (σ^{N}, RpoN, NtrA) (see Merrick, 1992; Fischer, 1994). An active NifA-, σ^{54}-dependent promoter, upstream from *nifH*, regulates transcription of the *nifHDK-orf1-nifY* operon (see Elmerich *et al.,* 1997). Similar putative NifA- and σ^{54}-dependent promoters were found upstream of *nifE*, *orf2*, and *nifB* (Liang *et al.,* 1992; Frazzon and Schrank, 1998; Potrich *et al.,* 2001a).

3.2. Nitrogen-Fixation Genes in Herbaspirillum seropedicae

H. seropedicae strain Z78, which is resistant to streptomycin, is named SMR1 and is the best-studied strain in terms of genetics, physiology, and molecular biology of nitrogen fixation. The sequencing of its genome is near completion (see www.genopar.com.br or www.genopar.org). The *nif* genes of *H. seropedicae* are found in two clusters located apart in the genome. Region I contains contiguous *modE, nifA* and an apparently large operon of *nifB fdxN hesB orf1 nifZ nifZ* orf4 nifS fixU orf5 orf6* (Figure 1). A single NifA-, σ^{54}-dependent promoter was found upstream of *nifB* and there was no evidence of an internal promoter. The promoter of *nifA* is described below. Region II contains the *nifHDKENX orf1 orf2 fer3* operon, followed by *nifQ modABC*, and then *fixABCX*; the last operon is transcribed in the opposite direction to *nifH* (see Souza *et al.,* 2000b; Pedrosa *et al.,* 2001; and the *H. seropedicae* genome-sequencing project). A NifA- and σ^{54}-dependent promoter was found upstream of *nifH*, but not upstream *nifQ*.

3.3. Nitrogen-Fixation Genes in Gluconacetobacter (Acetobacter) diazotrophicus

A 30.5-kb DNA fragment of *G. diazotrophicus* has been fully sequenced and 32 genes, including *mod*, *fdx*, *ynf*, *fix* and *mcp* genes, were found to be organized in 8 transcriptional units (Lee *et al.,* 2000; see Chapter 10). Potential NifA- and σ^{54}-dependent promoters occur upstream of *orf11, nifB, nifH, orf6*, and *fixA* (Lee *et al.,* 2000). A NifA-binding site was found upstream of *nifA*, but no σ^{54}-dependent promoter (Lee *et al.,* 2000). The operon that contains the genes coding for the nitrogenase proteins, *nifHDKENX orf4 fdxB orf1 nifQ*, has a structural organization similar to that of *H. seropedicae*, *i.e*., *nifHDKENX orf1 orf2,* where neither *nifT* nor *nifY* are present; a unique NifA- and σ^{54}-dependent promoter, which is upstream from *nifH*, regulates the synthesis of a single large transcript (Lee *et al.,* 2000; Machado *et al.,* 1996; Klassen *et al*., 1999).

3.4. Nitrogen-Fixation Genes in Azoarcus *BH72*

Two *nif*-gene clusters have been described in strain BH72 (see Chapter 9); one contains the structural genes for nitrogenase (n*ifHDK*), a ferredoxin gene (*fdxN*), a *nifY*-like gene, and *orf1* and the other has a *nifLA* operon (Egener *et al*., 2002). The presence of *nifL*-like genes is unusual in β–Proteobacteria. Hitherto, they have been found only in the γ–Proteobacteria (see Dixon, 1998) and, more recently, in *P. stutzeri* (Desnoues *et al*., 2003). The *nifL* gene has been found neither in *H. seropedicae*, a β–Proteobacteria, nor in the α–Proteobacterium *A. brasilense*, nor in the rhizobia. The structural genes of nitrogenase in strain BH72 constitute an operon that contains *nifHDK*, *fdxN, nifY,* and *orf1* and is transcribed from a NifA-dependent σ^{54} promoter (Egener *et al*., 2001).

3.5. Nitrogen-Fixation Genes in Pseudomonas stutzeri

A 30-kb region of *P. stutzeri* A1501 that contains the *nif* operons and flanking genes has been characterized (Desnoues *et al*., 2003). In this DNA region (Figure 1), 37 open reading frames were found and annotated. A *nifL* gene is associated with *nifA* as found in other member of the Proteobacteria γ–subgroup. Between *nifLA* and the *nifHDK* operon, the region contains a group of genes, *rnfABCDGEH*, initially identified in *Rhodobacter capsulatus* (Schmel *et al.,* 1993). These genes, which were described as playing a role in electron transfer to nitrogenase, code for transmembrane proteins that are similar to components of an NADH ubiquinone oxidoreductase; they have also been reported in non-nitrogen fixers (Schmel *et al.,* 1993; Kumagai *et al.,* 1997; Jouanneau *et al.,* 1998). The *rnf* genes have been found in *Azotobacter* (Rubio *et al.,* 2002) and *rnfA* is probably present in *Azoarcus* (Egener *et al.,* 2002). The organisation of the *nif* and *rnf* genes in *P. stutzeri* resembles that in *A. vinelandii,* although the *rnf* cluster is not located near *nifHDK* in the latter species (Rubio *et al.,* 2002). Another copy of a *nifY*-like gene is located in the vicinity of *rnfH* as occurs in *A. vinelandii* and is very similar to the *nafY* gene characterized in *Azotobacter* (Rubio *et al.,* 2002). Sequences reminiscent of σ^{54}-binding sites are present upstream from *nifL*, *rnfA*, and *nifH* (Desnoues *et al*., 2003). Construction of mutant strains, by insertion of kanamycin cassettes into *nif*- and *rnf*-coding sequences, led to Nif$^-$ strains showing that all are essential for nitrogen fixation in *Pseudomonas.*

3.6. Nitrogen-Fixation Genes in Other Systems

The organization of the *nif* genes was also studied in detail in *P. agglomerans* strains that were isolated from the rhizosphere of grasses from tropical or temperate countries. A striking feature was the plasmid location of the *nif* genes in these isolates (Steibl *et al.,* 1995). The gene order and organization resembled that of *K. pneumoniae* with the exception of *nifJ*, which is part of an operon with *nifF,* being located downstream of *nifQ* (X99694, X78558) (Siddavattam *et al.,* 1995 and ref.

therein). There is also an additional gene, *nifI*, associated with *nifU*, whose predicted translation product resembles to members of the HesB-protein family.

Occurrence of nitrogen-fixing *Bacillus* sp. in association with plants has been known for a long time among the Firmicutes (Döbereiner and Pedrosa, 1987). *B. polymyxa*, *B. macerans* and *B. azotofixans* have now been reassigned to a new genus, *Paenibacillus* (Achouak *et al.,* 1999). The genetic organization of a *P. azotofixans* strain involves three clusters, each of which contains a copy of *nifH*; these are (i) *nifB nifHDK*, (ii) *nifB nifH*, and (iii) *nifH* (Choo *et al*., 2003).

3.7. Multicistronic Operons and Translational Overlapping/Coupling

A recurrent feature of the structural organization of *nif* and related genes in *A. brasilense, G. diazotrophicus, P. stutzeri* and *H. seropedicae* is the parsimony in the number of identified promoters leading to the presence of large multicistronic operons and suggesting very large transcripts. The linkage of a high number of genes is conserved as is the translational overlapping between the 3' and 5' ends. Translational overlapping is indicative of translational coupling.

For example, in *G. diazotrophicus*, the *nifN-nifX* (30 bp), *fixC-fixX* (1 bp), *rpoN-modC* (3 bp), *modC-modB* (3 bp), *modB-modA* (3 bp), *nifZ-fixU* (3 bp), and *fixU-orfl* (3 bp) have translational overlapping (Lee *et al.,* 2000). Translational coupling has also been found between *nif* and/or related genes in other diazotrophs, including *K. pneumoniae, A. vinelandii*, and the Archaea diazotroph *Methanococcus maripaludis* (see Lee *et al.,* 2000 and references therein), as well in *P. stutzeri* (Desnoues *et al*., 2003) and *A. brasilense* (Ishida *et al.,* 2002).

Translational coupling and large operons may reflect the over-packing present in bacterial genomes, possibly contributing to cellular economy, and probably tight regulation of gene expression and mRNA translation. Translational coupling thus prevents independent translation of distal genes in transcripts and maintains the stoichiometry of translated products (see Govantes *et al.,* 1998). In the *nifLA* operon, it has been shown to be important in maintaining a stoichiometric level of NifL and NifA, which is essential for the regulatory role of NifL (Govantes *et al.,* 1998, see references therein).

4. IDENTIFICATION OF RPON AND ITS INVOLVEMENT IN NITROGEN FIXATION

The RNA polymerase sigma factor, σ^{54} (RpoN, NtrA, σ^{N}), is encoded by the *rpoN* gene and is involved in the recognition of -24/-12 promoters (σ^{54}-dependent promoters). These promoters have the consensus sequence 5'-TGGCAC-N_5-TTGCA/T, with the GG and GC being highly conserved, and can be used as a first approach diagnostic for this promoter type (Barrios *et al.,* 1999; Buck *et al.,* 2000). The σ^{54}-dependent promoters require positive transcription activator proteins (*e.g*., NifA, NtrC), which respond to specific environmental signals and which bind to specific upstream activator sequences (UAS) located at about –130 bp from the transcription start (see Barrios *et al.,* 2000; Buck, 2000; and references therein).

4.1. Pleiotropic Role of rpoN

The expression of a large number of metabolic functions of Proteobacteria is regulated by such promoters, including the utilization of alternative carbon (dicarboxylate transport) and nitrogen sources (nitrogen fixation, nitrate utilization, nitric oxide, NH_4^+ assimilation, amino-acid utilization), flagellar synthesis, cell motility, O-antigen expression, hydrogenase synthesis, and the expression of virulence determinants (Kustu *et al*., 1989; Fischer *et al.,* 1994; Barrios *et al.,* 1999). The number of metabolic functions dependent on RpoN increases continually.

4.2. The rpoN *Gene in Associative and Endophytic Bacteria*

As described above, the σ^{54}-type promoters are located upstream of their subject genes and have been shown to regulate the expression of *nif* and *fix* operons of *A. brasilense, H. seropedicae*, *G. diazotrophicus*, *Azoarcus* BH72, and *P. stutzeri* after activation by either NifA or NtrC. The *rpoN* genes of A. *brasilense* (Milcamps *et al.,* 1996), *G. diazotrophicus* (Lee *et al.,* 2000), *H. seropedicae* (Genome project), and *P. stutzeri* (AJ496594) have been sequenced. No *rpoN* genes have as yet been isolated or sequenced from *Azoarcus*.

An *rpoN* mutant of *A. brasilense* Sp7 was found to be defective in nitrogen fixation, nitrate assimilation, ammonium uptake, and flagellar biosynthesis (Milcamps *et al.,* 1996). In *P. stutzeri*, the nucleotide sequence of the the DNA region downstream of *rpoN* revealed four orfs (orf2, *ptsN*, orf4 and *ptsO*) that are commonly found at the same location in γ–Proteobacteria (Jones *et al.,* 1994 and refs. therein). Nitrogenase activity of *ptsN* and *ptsO* mutant strains was decreased. Inactivation of *rpoN* led to a Nif^- mutant that was impaired in several carbon and nitrogen sources and devoid of flagella (Ma Luyan, Carreño-Lopez, Desnoues, and Elmerich, unpublished results). This mutant is also strongly impaired in the root-surface colonization of rice (Carreño-Lopez *et al*., 2003).

5. THE NTR SYSTEM AND THE CONTROL OF NITROGEN METABOLISM AND NITROGEN FIXATION

In general, ammonium is the preferred nitrogen source for Gram-negative bacteria. The first step in its assimilation is the transport across the bacterial membrane by either free diffusion or active transport (Kleiner, 1985). The primary route for NH_4^+ incorporation into organic forms is the synthesis of glutamate and glutamine, which are the precursors of other nitrogenous compounds in the cell (Prusiner and Stadtman, 1973). Glutamine synthetase (GS), encoded by *glnA*, and glutamate synthase (GOGAT), encoded by *gltBD*, are the key enzymes in NH_4^+ assimilation in diverse species.

The ntr system involves both *ntr* and *gln* genes, whose products act as a multi-tier transcriptional/post-translational regulatory cascade that is responsive to fluctuations in NH_4^+ levels (reviewed by Magasanik, 1988; Merrick and Edwards, 1995; Arcondéguy *et al.,* 2001). The uridylyltransferase/uridylylase (UTase or

GlnD, the product of *glnD*) is the primary nitrogen sensor. The small trimeric protein, PII, which is encoded by *glnB*, is an intracellular signal transmitter that coordinates the response of the Ntr system with the N-status of the cell. Two other proteins, NtrB (the product of *ntrB*), which is a histidine kinase, and NtrC (the product of *ntrC*), which is a transcriptional activator, belong to a two-component regulatory system. Alternative participants are the PII-protein paralogues, GlnK (the product of *glnK*), GlnZ (the product of *glnZ*), GlnY (the product of *glnY*) (see Arcondéguy *et al.,* 2001), and a sensor/regulatory pair, NtrY/NtrX, of which NtrY is a transmembrane histidine kinase/phosphatase and the product of *ntrY* and NtrX is a transcription activator and the product of *ntrX* (Vitorino *et al.,* 2001; Ishida *et al.,* 2002).

5.1. Pathways for NH_4^+ Assimilation

5.1.1. Ammonium Transport

Protonated NH_4^+ is in equilibrium with the uncharged NH_3. Bacterial membranes are permeable to small, uncharged compounds and NH_3 rapidly passes through membranes by unspecific diffusion. Under nitrogen-limiting conditions, there is a requirement for an energy-dependent uptake system to maintain a sufficient NH_4^+ concentration inside the cells (Kleiner, 1985). Active uptake is usually measured by accumulation of radioactive methylammonium (Kleiner, 1985). Physiological evidence for NH_4^+ (methylammonium) carriers has been established in a number of nitrogen-fixing bacteria, including *Azospirillum, Azotobacter, Clostridium pasteurianum, K. pneumoniae, Rhizobium*, and cyanobacteria (Hartmann and Kleiner, 1982; Kleiner, 1985; Shehawy and Kleiner, 1999).

In an early work with *K. pneumoniae,* a mutant strain with an Amt^- phenotype, which was unable to grow at 1 mM NH_4^+ but capable of growth with 20 mM NH_4^+, was impaired in its NH_4^+ carrier. The Amt^- strain excreted NH_3 into the medium when grown with N sources other than NH_4^+. This observation supported a role of the NH_4^+ carrier in the maintenance of intracellular pools of NH_4^+ under N-limitation conditions (Kleiner, 1985). In *A. brasilense*, mutants isolated as resistant to methylammonium and ethylenediamine were found impaired in NH_4^+ transport (Turbanti *et al*., 1988; Machado *et al*., 1990). Some of these mutants were also Nif constitutive (*i.e*., fixing nitrogen in the presence of ammonia, Nif^c). Gauthier and Elmerich (1977), when isolating glutamine auxotrophs, obtained also a class of Nif^c mutants, which were found later to excrete ammonia and to be deficient in methylammonium uptake (Hartmann *et al*, 1984). Interestingly, those mutants had an altered adenylylation level of GS (Gauthier and Elmerich, 1997; Machado *et al*., 1990; see section 5.1.4)

Characterization of putative genes for the NH_4^+ carrier (*amt*) in plants, yeast and bacteria showed they belong to a conserved family that encoded membrane-anchored proteins (Meletzus *et al.,* 1998; Van Dommelen *et al.,* 1998; Coutts *et al*., 2002). In most bacteria, the *amtB* gene is linked to *glnK*, which is a *glnB*-like gene (reviewed by Arcondéguy *et al.* 2002; see section 5.3.2); an exception is found in *A. brasilense* (Van Dommelen *et al*., 1998). There is clear evidence for the

involvement of AmtB in NH_4^+/methylammonium uptake, *e.g*., in *A. vinelandii*, *A. brasilense*, and *Azoarcus*. However, in these bacteria, *amtB* mutants were not severely impaired in growth at low NH_4^+ concentrations, suggesting existence of alternative, not yet identified, NH_4^+ carriers (Meletzus *et al.,* 1998; Van Dommelen *et al*., 1998; Martin and Reinhold-Hurek, 2002). In *A. brasilense*, the expression of an *amtB-gusA* fusion was repressed by high NH_4^+ concentrations and depended on *rpoN* and *ntrBC* genes (Van Dommelen *et al*., 1998). In addition, methylammonium transport is increased in a *glnZ* (*glnK*) mutant, which suggests that GlnZ (GlnK) acts as a negative regulator of AmtB activity (de Zamaroczy, 1998; see section 5.3).

The purification and properties of the AmtB protein was achieved in *Escherichia coli*. The native protein is a trimer composed of 44.5-kDa subunits (Blakey *et al*., 2002). A direct interaction between GlnK and AmtB was demonstrated. In addition, GlnB could also interact with AmtB, particularly in the absence of GlnK (Coutts *et al*., 2002). The AmtB C-terminal domain is involved in the sequestration of GlnK in the membrane, and the level of sequestration depends on the uridylylation state of GlnK. Thus, the concentration of GlnK in the cytoplasm decreases after ammonia shock. Then, under N limitation when GlnK is fully uridylylated, there is little interaction with AmtB; AmtB then displays its NH_4^+-uptake activity and GlnK is found essentially free in the cytoplasmic fraction (Coutts *et al*., 2002).

5.1.2. Synthesis of Glutamate and Glutamine

In most cases, bacteria possess a NAD(P)H-dependent glutamate dehydrogenase (GDH), which catalyses direct amination of α-ketoglutarate (2-KG) through the following reaction.

$$\text{2-KG} + NH_4^+ + \text{NAD(P)H} + H^+ \leftrightarrow \text{glutamate} + \text{NAD(P)}^+$$

However, due to its high K_m for NH_4^+, this enzyme plays a minor role under nitrogen-limiting conditions. The main assimilation route results from the coupling of the ATP-dependent glutamine synthetase (GS) and the glutamate synthase (GOGAT) activities (Prusiner and Stadtman, 1973). GOGAT, a NADPH-dependent enzyme, catalyzes the reductive transfer of the glutamine amide group to 2–KG to yield two molecules of glutamate (Meers *et al.,* 1970; Elmerich and Aubert, 1971).

$$\text{glutamine} + \text{2–KG} + \text{NADPH} + H^+ \rightarrow 2\ \text{glutamate} + \text{NADP}^+$$

GS in turn catalyzes the ATP-dependent amination of glutamate to glutamine.

$$\text{glutamate} + NH_4 + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + \text{Pi}$$

In this system, glutamine is the nitrogen donor for glutamate synthesis. The functioning of the two enzymes as a cycle accounts for the incorporation of all the inorganic nitrogen into the cell.

5.1.3. Regulation of the GS Activity by Covalent Modification

In most Gram-negative bacteria, ammonia shock leads to a rapid decrease in GS activity due to covalent modification of the GS subunits by adenylylation and GS activity is controlled by cumulative feedback inhibition by the metabolites for which glutamine is the amino-group donor (Prusiner and Stadtman, 1973).

Post-translational modification of GS in response to the fixed-nitrogen concentration is a complex regulatory process that has been extensively documented (Stadtman and Prusiner 1973; Magasanik, 1988; Merrick and Edwards 1995; Arcondéguy *et al.,* 2001 and references therein; Figure 2). A tyrosine residue of each GS subunit can be adenylylated by an adenylyltransferase (known as ATase or GlnE, the product of *glnE*) and the extent of adenylylation depends on physiological conditions. The signals that turn on or off adenylylation are sensed by the *ntr* system (see section 5.2). It involves both GlnD and PII.

As shown in Figure 2, GlnD, a dual-activity enzyme, uridylylates PII when the ratio 2-KG/Gln is high (conditions of fixed-nitrogen deficiency) and deuridylylates PII-UMP when this ratio is low (conditions of nitrogen excess). Under N-limitation, PII-UMP complexes to and confers adenylylase activity to GlnE. The GlnE/PII-UMP complex catalyzes AMP removal from glutamine synthetase, which leads to its activation. When fixed-N is in excess, GlnE and PII adenylylate GS, which leads to its inactivation.

5.1.4. The Importance of GS and GOGAT in the Regulation of the Nitrogen Metabolism in Azospirillum

GS and GOGAT are the two key enzymes of ammonia assimilation and glutamate synthesis in *A. brasilense* (Westby *et al.,* 1987). Both enzymes have been characterized (Ratti *et al.,* 1985; Colombo Pirola *et al.,* 1992). GS a dodecamer of M_r 630,000 is composed of identical subunits of 52,000 similar to the *E. coli* enzyme (Colombo Pirola *et al.,* 1992). In *A. brasilense*, the maximal level of adenylylation was observed in cultures grown in the presence of ammonia, whereas the minimal level was observed under conditions of N_2 fixation. GS synthesis was repressed from 2- to 8-fold by high ammonia concentrations (Okon *et al.,* 1976; Gauthier and Elmerich, 1977; Westby *et al.,* 1987; Colombo Pirola *et al.,* 1992). The GS has also been characterized from *G. diazotrophicus.* In this strain, GS activity is regulated by covalent modification, but its synthesis is not regulated by ammonia availability (Ureta and Nordlund, 2001).

Isolation of glutamine auxotrophs in *A. brasilense* led to pleiotropic mutants impaired both in GS activity and in the regulation of nitrogen fixation, some of which were Nif^- and others Nif^c (Gauthier and Elmerich, 1977). The Nif^c mutants were also capable of ammonia excretion and their GS was fully adenylylated (Gauthier and Elmerich, 1977; Hartmann *et al.*, 1984; Machado *et al.,* 1990; Van Dommelen *et al.*, 2003). Genetic complementation for the wild-type phenotype led to the isolation of the structural gene of the GS, *glnA*, suggesting that those mutants might be *glnA* mutants and that GS played a regulatory role in nitrogen fixation (Bouzouklian *et al.*, 1986). More recently, Van Dommelen *et al.* (2003) showed, after PCR amplification of the *glnA* gene from the mutants and establishment of the nucleotide sequence, that they carried point mutations in *glnA*.

GOGAT's biochemical properties have been extensively studied in *A. brasilense* (Vanoni and Curti, 1999). The native enzyme is a flavoprotein (Mr 740 kDa) composed of two subunits (Mr of 164 and 52 kDa). It has a $\alpha_4\beta_4$ quaternary

structure, similar to that of the *E. coli* enzyme (Ratti *et al.,* 1985; Stabile *et al*., 2000). The nucleotide sequence of the *gltB* and *gltD* genes, which encode the GOGAT large and small subunits, respectively, was established and the translation products revealed high identity to those of *E. coli*. Interestingly, the *gltD* gene precedes *gltB*, in contrast to the situation in *E. coli* (Pelanda *et al.,* 1993). In *E coli*, and in several nitrogen-fixers, *gltBD* is co-transcribed with *gltF*. This latter gene was proposed to play a regulatory role in the nitrogen metabolism, because mutants that were impaired in GOGAT activity and that map in the *glt* region were found to display a regulatory phenotype of the *ntr* type, *i.e*., being impaired in nitrogen sources catabolism (Castano *et al.,* 1992). There is no *gltF* gene in *Azospirillum,* although GOGAT-deficient mutants were found impaired in nitrogen fixation and for growth on nitrate and several other nitrogen sources (Bani *et al*., 1980; reviewed in Döbereiner and Pedrosa, 1987). The regulatory role of *gltF* in *E. coli* was further denied after specific interruption of *gltF* (Grassl and Kleiner, 1999).

It thus appears that deprivation of the glutamate pool in *Azospirillum* caused by GOGAT inactivation and changes in the catalytic activity of GS strongly impair the nitrogen metabolism of the cell and the regulation of nitrogen fixation.

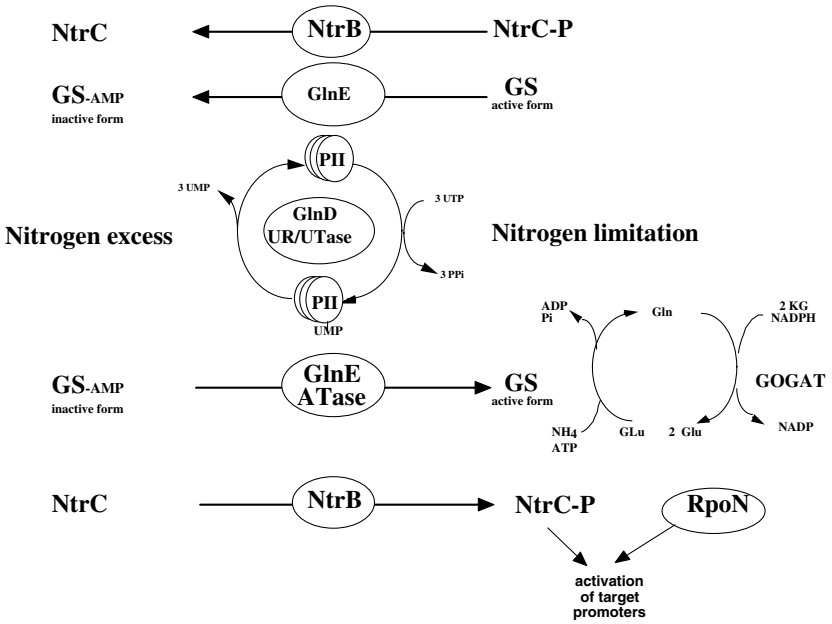

Figure 2. Schematic representation of the Ntr system showing adenylylation of GS and assimilation of ammonium ions. See text for details.

5.2. The ntr *Control*

The involvement of GlnD and PII is not limited to the regulation of GS activity, in fact, they play a major role in the control of nitrogen-source utilization through the modulation of NtrBC activity (Magasanik, 1988; Merrick and Edwards 1995; Arcondéguy *et al.,* 2001; Figure 2). The *ntrBC* genes encode a two-component regulatory system. NtrB, a histidine kinase, can phosphorylate NtrC in response to fixed-nitrogen limitation. PII-UMP is unable to complex NtrB, which in the free form is capable of phosphorylating NtrC to NtrC-P, the transcriptionally active form. In its phosphrylated form, NtrC-P acts as a transcriptional activator of a number of operons involved in nitrogen-sources utilization, ammonia assimilation, transcription of the *nifLA*, and other NtrC-/σ^{54}-dependent promoters (see Merrick and Edwards, 1995, for a review). As a result, multiple operons that are capable of mobilizing alternative NH_4^+ sources, such as N_2, NO_3^-, amino acids, purines, *etc*, are simultaneously transcribed with thte aim of replenishing the cell with NH_4^+.

Alternatively, under conditions of NH_4^+ sufficiency, glutamine activates GlnD to deuridylylate PII-UMP, which in its PII form activates GlnE and NtrB to adenylylate glutamine synthetase (see above) and dephosphorylate NtrC-P, respectively, so inactivating both proteins (Merrick and Edwards, 1995; Arcondéguy *et al.,* 2001). This prevents transcription of NtrC-/σ^{54}-dependent promoters, such as the *nifLA* of *K. pneumoniae* and probably those of *Azoarcus BH72* and *P. stutzeri*.

5.3. Organization of the ntr *System Genes in Associative and Endophytic Bacteria*

5.3.1. The ntrBC/XY *Genes*

The *ntrBC* genes are linked to *nifR3* in the *nifR3-ntrB-ntrC* operon in *A. brasilense* (Liang *et al.,* 1993; Machado *et al.,* 1995). Downstream from this operon is the *ntrYX* operon (Ishida *et al.,* 2002). A similar gene organization is found in *G. diazotrophicus* (Accession number AF494454). The *A. brasilense nifR3-ntrB-ntrC* promoter, located upstream from *nifR3*, contains two sequences, one resembling the σ^{70}- and one the σ^{54}–consensus promoter. Its expression is independent of RpoN and ammonium levels, but is negatively regulated by NtrC (Machado *et al.,* 1995). An NtrC-/σ^{54}-dependent promoter that is regulated by ammonia was found upstream of *ntrC* and inside the *ntrB* gene (Liang *et al.,* 1993). The probable translational coupling in NtrY/NtrX of both *A. brasilense* and *G. diazotrophicus* may suggest that NtrY and NtrX must be synthesized in a stoichiometric ratio that could be essential for the role of the trans-membrane NH_4^+ sensor, NtrY.

In *H. seropedicae*, the *ntrBC* genes are linked to *glnA* in a *glnA-ntrB-ntrC* operon (Accession number AF0828730) as in the diazotrophs, *K. pneumoniae* (Espin *et al.,* 1982) and *A. vinelandii* (Toukdarian and Kennedy, 1986). Sequences similar to NtrC-binding sites, σ^{54}- and σ^{70}-dependent promoters are found upstream from *glnA*, with no known promoters in the intergenic regions. Studies involving *lacZ* fusion and *ntrC* mutants suggest that the upstream region of *glnA* of *H. seropedicae* contains two active promoters, one dependent on NtrC (σ^{54}-type) that is

operational under N-limitation and another, a putative σ^{70} promoter, that is active under NH_4^+ -excess (Persuhn *et al.,* 2000).

The *ntrBC* region has been characterized in *P. stutzeri*, and is not associated with a *glnA* gene. The use of a *lacZ* fusion showed that *ntrBC* expression was controlled by *rpoN* and that NtrC was required for its own synthesis. An unusual feature, not reported in other systems, that may reflect a particular and different regulatory cascade, was the requirement of NifA for full expression of *ntrBC* (Desnoues *et al.,* 2003).

5.3.2. The PII Protein Family: GlnB, GlnK, GlnZ and GlnY

Three major groups of PII proteins, GlnB, GlnK and NifI were defined from structural and functional relationships by Arcondéguy *et al.* (2001). The prototype PII protein is encoded by the *glnB* gene of *E. coli*. The second group contains the *glnK*-like proteins also originally described in *E. coli* and frequently occurring linked to the ammonium-transport gene, *amtB*. The recent demonstration of the sequestration of GlnK by the membrane-bound AmtB in *E. coli* and *A. vinelandii*, under conditions of NH_4^+ excess (Coutts *et al*., 2002), apparently in a 1:1 ratio, suggests a control of gene expression by translational coupling. The third group, coded by *nifI,* is found in methanogens (see Arcondéguy *et al.,* 2001 and references therein). In the associative/endophytic diazotrophs, there are representatives of two of the three major groups of prokaryotic PII proteins, GlnB and GlnK.

In *A. brasilense*, one copy of *glnB* is linked to *glnA* (operon *glnBA)*, as in other α–Proteobacteria, including *G. diazotrophicus* (Arcondéguy *et al.*, 2001 and references therein). The other copy, named *glnZ* (product Pz*),* is a *glnK*-like gene, but is monocystronic and not linked to *amtB* (De Zamaroczy *et al.,* 1996; De Zamaroczy, 1998). Expression of the *glnBA* operon is regulated by three NH_4^+-responsive promoters, both σ^{70} and σ^{54} promoters that are located upstream of *glnB* and a third promoter located upstream of *glnA*. Under condition of ammonia sufficiency, both the σ^{70} and the *glnA* promoters are active at low levels, whereas under conditions of NH_4^+ limitation, the σ^{54} promoter is activated and transcription of the *glnBA* operon increases five-fold (De Zamaroczy *et al.,* 1993). The two-component regulatory system, NtrB/NtrC, is not required for the expression of this operon (De Zamaroczy *et al*., 1993; De Zamaroczy, 1998). In contrast, transcription of *glnZ* (*glnK*-like) is dependent on NtrC and σ^{54} (De Zamaroczy, 1998). Both PII and Pz are uridylylated under N deficiency (De Zamaroczy, 1998).

There is evidence for two *glnB*-like genes in *H. seropedicae*, one has been sequenced (Benelli *et al.,* 1997) and had its three-dimensional structure determined (Benelli *et al.,* 2002). This *glnB*-like gene is linked to a gene, *nadE*, which codes for an NH_4^+-dependent NAD synthase (Benelli *et al.,* 1997). Although structurally related to the GlnK of *E. coli* (Benelli *et al.,* 2002), the structural organization and expression of the *H. seropedicae glnB* (*glnK*-like) is distinct from other *glnK* genes because it is independent of NH_4^+, NtrC and σ^{54}.

Three PII-like proteins were identified in *Azoarcus* sp. BH72; one is encoded by *glnB* and monocistronically transcribed, whereas the two other *glnK*-like genes are part of the *glnK-amtB* and *glnY-amtY* operons (Martin *et al.,* 2000). NH_4^+ limitation

enhanced transcript abundance of *glnK* strongly, *glnY* moderately, and *glnB*; neither GlnK nor GlnB were essential for nitrogen fixation, a function suggested to involve GlnY (Martin *et al.,* 2000). All three PII-like proteins were uridylylated under NH_4^+ limitation, as all GlnB- and GlnK-like proteins are. GlnY was unusual because it was only detected in a *glnBK* double mutant and only occurred in the uridylylated form (Martin *et al.,* 2000). Nitrogenase activity and a *nifH-gusA* were equally expressed in the wild type and in the *glnB*, *glnK* or *glnBK* mutants, suggesting that these PII-like proteins could substitute for each other under nitrogen-fixing conditions (Martin and Reinhold-Hurek, 2002).

Three *glnB*-like genes have been identified in *G. diazotrophicus*; one is linked to *glnA* (*glnBA*) and the two other to *glnK* (*glnK1-amtB1* and *glnK2-amtB2* operons) (Perlova *et al.,* 2003; see Chapter 10). The *glnK1-amtB1* and *glnK2-amtB2* operons of *G. diazotrophicus* also display translational overlapping/coupling.

P. stutzeri has an unusual *glnK-amtB1-amtB2* gene cluster, encoding the PII homologue/paralogue GlnK and two putative ammonium transporters, AmtB1 and AmtB2. These are probably transcribed from a single NtrC-/σ^{54}-dependent promoter located upstream of *glnK* and regulated by ammonia (Vermeiren *et al.,* 2002).

5.4. Role of ntrBC *and PII in Regulation of Nitrogen Fixation and Assimilation*

5.4.1. Azospirillum brasilense

The NtrB/NtrC sensor/regulatory pair is not essential for nitrogen fixation *in A. brasilense* because single and double mutants are Nif^+, showing around 60% of the wild-type nitrogenase activity; they are, however, required for full nitrogenase activity (Liang *et al.,* 1993; Machado *et al.,* 1995). The NtrB/NtrC pair is required for both nitrate-dependent growth and NH_4^+-triggered nitrogenase switch-off in *A. brasilense* (Liang *et al.,* 1993; Elmerich *et al.,* 1997). The NtrY/NtrX pair is interchangeable with the NtrB/NtrC pair for nitrate-dependent growth and regulation of *glnB* expression or activity (Vitorino *et al.,* 2001).

The finding that a *glnB* mutant was Nif^- was particularly puzzling (Liang *et al.,* 1992), considering that *ntrBC* are not essential for nitrogen fixation and that PII is required neither for GS synthesis nor for GS covalent modification (De Zamaroczy *et al.,* 1993). Moreover, *nifA* expression was not altered in a *glnB* mutant (Liang *et al.,* 1992). It was thus hypothesized that PII was essential for nitrogen fixation and was involved in NifA activation. This hypothesis was further documented (Arsène *et al*., 1996; Arsène *et al.,* 1999; detailed in section 6.4). Inactivation of *glnZ* resulted in a mutant strain that differed from the *glnB* mutant, suggesting that Pz and PII, although very similar, have different functions. Thus, the *glnZ* mutant was neither impaired in nitrogen fixation nor in motility in contrast to the *glnB* mutant. Pz, as with PII, was not required for GS covalent modification. A specific role was, however, found for Pz; it negatively regulates (methyl)ammonium uptake (De Zamaroczy, 1998) and is required for restoration of activity after NH_4^+-induced nitrogenase switch-off (Klassen *et al.,* 2001; see section 6.1.1). A double mutant in *glnB* and *glnZ* was severely impaired in growth properties in rich medium, suggesting that either PII or Pz can have a common role in cell growth (De

Zamaroczy, 1998). A *glnD* mutant of *A. brasilense* was found to be Nif$^-$ (Van Dommelen *et al*., 2002).

5.4.2. Herbaspirillum seropedicae

H. seropedicae ntrB/ntrC mutants were Nif$^-$ (see Pedrosa *et al*., 2001). A *glnB* mutant was also Nif$^-$, but it was able to synthesize NifA (Pedrosa *et al.,* 1998), as reported for *A. brasilense*, implying that the second *glnB*-like gene is unable to substitute for *glnB* in activating NifA under nitrogen-fixing conditions (Benelli *et al.,* 1997). In addition, PII is not involved in the adenylylation/deadenylylation control of GS activity (Benelli *et al.,* 1997; 2001). Purified PII protein of *H. seropedicae* is capable of reversible uridylylation either by purified *E. coli* GlnD or by a cell-free extract of *H. seropedicae* (Benelli *et al.,* 2001). Urydylylation was stimulated by α-ketoglutarate and ATP and inhibited by glutamine. Deuridylylation of PII-UMP was dependent on glutamine and inhibited by ATP and α-ketoglutarate (Benelli *et al*., 2001).

6. REGULATION OF NITROGEN FIXATION

The high energy-demanding reduction of dinitrogen to ammonium (nitrogen fixation) is a strictly regulated process. Under condition of ammonium sufficiency and/or excess O_2, nitrogenase synthesis is repressed and, in most cases, its activity rapidly ceases due to inactivation, all of which lead to a saving of energy and plastic reserves. In all diazotrophic Proteobacteria, regulation of nitrogen fixation is centered on the control of the synthesis and activity of NifA, a transcriptional activator of σ^{54}-dependent promoters (see Merrick, 1992; Merrick and Edwards, 1995; Fischer, 1994; Arcondéguy *et al.,* 2001).

6.1. Regulation of Nitrogenase Activity by Reversible Inactivation (Switch-on/off)

Efficient short-term mechanisms for reversibly inactivating the high energy-demanding nitrogenase have been evolved by several diazotrophs. The best-characterized mechanism involves ADP-ribosylation of the Fe-protein component (also known as dinitrogenase reductase or component 1 or NifH dimer) by dinitrogenase reductase ADP-ribosyl transferase (DraT) when triggered by sudden changes in fixed-nitrogen concentration (NH_4^+ or glutamine), by anaerobiosis, or by darkness, depending on the organism (Ludden, 1994). The inactivation results from the covalent modification of an Arg residue of one NifH subunit (Figure 3). Reactivation is due to dinitrogenase reductase activating glycohydrolase (DraG). This mechanism, with species-specific elicitors, is present in several Proteobacteria of the *Rhodospirillaceae*, such *A. brasilense, A. lipoferum, Rhodospirillum rubrum*, and *Rhodobacter capsulatus* (Ludden, 1994; Zhang *et al.,* 1997; Arcondéguy *et al.,* 2001, and references therein) and also in *Azoarcus*. Other mechanisms of inactivation without apparent covalent modification have been reported (see section 6.1.2). The same species can display several inactivation mechanisms.

6.1.1. Inactivation by ADP-Ribosylation

In *A. brasilense* and *A. lipoferum*, nitrogenase switch-off is triggered by NH_4^+, glutamine or anaerobiosis, all of which lead to ADP-ribosylation of the Fe protein (Zhang *et al.,* 1997). Recovery of activity occurs, as in other systems, following either exhaustion of fixed-N or return to O_2-limiting conditions. The *draTG* operons of both *Azospirillum* species have been sequenced and found to be located upstream from the *nifHDK* operon and transcribed divergently and constitutively (see Zhang *et al.,* 1997, and references therein).

Nitrogenase activity and ammonia-induced nitrogenase switch-off kinetics in a *glnZ* mutant of *A. brasilense* (strain 7611) were similar to those of the wild-type strain FP2 (Sp7 Nal^R Sm^R), however, the *glnZ* mutant failed to recover nitrogenase activity after NH_4^+ exhaustion (Klassen *et al.,* 2001). These results imply that DraG regulation is abolished, whereas DraT activity is not affected in the *glnZ* mutant. Anoxic-induced switch-off/on had the same kinetics in both the *glnZ* and wild type strains, suggesting the presence of two independent mechanisms for nitrogenase reactivation in *A. brasilense* (Klassen *et al.,* 2001). The involvement of GlnB (PII) in the switch-off mechanism, *i.e*., in DraT regulation, is documented for *R. rubrum* and *R. capsulatus* (see Arcondéguy *et al.,* 2001, for a review).

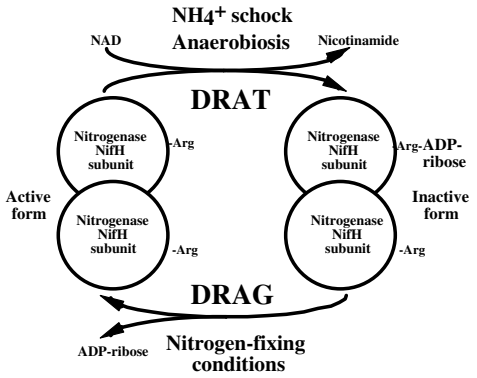

Figure 3. Model for inactivation/reactivation of nitrogenase activity by covalent modification of dinitrogenase reductase NifH subunit (see text for details).

Nitrogenase activity in *Azoarcus* BH72 is rapidly inactivated after addition of NH_4^+ to cultures and also after anaerobic shock (Egener *et al.*, 2001). Different inactivation mechanisms are involved (see section 6.1.2). A putative *draT* gene, encoding a DraT protein with 30% amino-acid identity to that of *A. brasilense,* has been found upstream of *nifH*, suggesting a modification of NifH by ADP-ribosylation in *Azoarcus* (Martin and Reinhold-Hurek, 2002). The role of GlnB and parologues in the nitrogenase switch-off and NifH covalent modification is complex. Martin and Reinhold-Hurek (2002) found it required both GlnB and

GlnK, but GlnY has no role. Nitrogenase inactivation by NH_4^+ required the putative high-affinity ammonium transporter, AmtB, and GlnK, but not GlnB. AmtB probably acts as a sensory component of NH_4^+ concentration. However, NifH covalent modification induced by NH_4^+ required GlnB in addition to GlnK and AmtB. Anaerobiosis-induced ADP-ribosylation required either GlnB or GlnK and not AmtB. A double *glnB/glnK* mutant did not show NifH covalent modification at all (Martin and Reinhold-Hurek, 2002).

6.1.2. Other Mechanism(s)

A second mechanism for the reversible nitrogenase switch-on/off, triggered by ammonium ions, but without covalent ADP-ribosylation of the Fe protein has been reported for *A. amazonense, A. vinelandii, G. diazotrophicus, H seropedicae, Anabaena variabilis,* and *Rhodobacter sphaeroides* (see Zhang *et al.,* 1997, and references therein). Nitrogenase activity switch-on/off was also reported in *P. stutzeri*, but it is not known if ADP-ribosylation is involved (Desnoues *et al.,* 2003). The absence of ADP-ribosylation may reflect the absence of the draTG genes in these organisms.

This second mechanism probably involves short-term membrane depolarization by ammonium ions, leading to an alteration both in electron supply to nitrogenase and in its redox state, as originally suggested for *A. vinelandii* (Laane *et al.,* 1980). After modification of the arginine residue (Arg 101) of the Fe protein, this second mechanism was also found to be operational in *A. brasilense* (Zhang *et al.,* 1996). The requirement for an active ferredoxin-N, product of *fdxN*, for nitrogenase switch on/off in *Azoarcus* (Egener *et al.,* 2001) appears to lend support to the involvement of electron flux in this mechanism. Ferredoxin-like genes are present in the *nif/fix* clusters of *H. seropedicae, G. diazotrophicus,* and *A. brasilense*; their gene products may be involved in the second DraT/DraG-independent mechanism of nitrogenase switch-on/off.

6.2. Regulation of the Synthesis of the NifA Protein.

6.2.1. Promoters of nifLA *Operons.*

In the γ–Proteobacteria*, K. pneumoniae* (Arnold *et al.,* 1988), *P. stutzeri* A1501 (Desnoues *et al*., 2003), *P. agglomerans* (Siddavattam *et al.,* 1995), *E. cloacae* (Deng and Shen, 1995), *A. vinelandii* (Blanco *et al.,* 1993), and the β–Proteobacterium, *Azoarcus* BH72 (Egener *et al.,* 2002), the *nifA* gene is located downstream from *nifL* to constitute a single *nifLA* operon. The *nifL* promoters of *K. pneumoniae* as well as that of *P. agglomerans* are of the σ^{54} type and require NtrC as the transcriptional activator (Minchin, *et al.,* 1989; Dixon 1998, and references therein). The *nifL* promoter of *Azoarcus* BH72 complies with this structure but has, in addition, a putative *nifA*-binding site upstream from two overlapping NtrC-binding sites; there is no evidence for a role for NifA in the regulation of this promoter (Egener *et al.,* 2002). The *nifL* promoter of *P. stutzeri* has not been yet identified, although it was shown that a *nifLA-lacZ* fusion expression depends on RpoN and NtrC and is controlled by O_2 and ammonia (Desnoues *et al*., 2003).

6.2.2. Promoters of nifA *in α– and β–Proteobacteria*

In the α–Proteobacteria, *A. brasilense* and *G. diazotrophicus*, and in the β–Proteobacterium, *H. seropedicae*, the *nifA* genes are monocystronic and located immediately upstream from *nifB*, with unique upstream regulatory structural elements.

The *nifA*-gene promoter of *A. brasilense* is atypical because it contains neither upstream NtrC- (nor NifA-) nor σ^{54}-binding sites (Liang *et al.,* 1991). Hence, its expression was independent of NtrB/NtrC. The *nifA* gene of *A. brasilense* was fully expressed under limited NH_4^+ and O_2, *i.e*., under conditions of nitrogen fixation, however, it was partially repressed by NH_4^+ under limited O_2 and almost fully repressed (80-90%) under high NH_4^+ and O_2 concentrations (Liang *et al.,* 1991). Newer observations from chromosomal *nifA-lacZ* fusions point to a synergistic repressive effect of O_2 and ammonium ions on *nifA*-promoter expression (Fadel-Picheth *et al*., 1999), confirming previous observations (Liang *et al.,* 1991).

Recently, a region essential for *nifA*-promoter activity was identified between nucleotides -67 and -47 from the transcription-start site. A sequence reminiscent of a σ^{70}-type promoter is present therein plus sequences resembling UP elements (Fadel-Picheth *et al.*, 1999). The presence of two sequences, which resemble an Fnr consensus sequence downstream from the transcription start, suggests that O_2 repression of *nifA* may involve an Fnr-like protein (Fadel-Picheth *et al.,* 1999).

The *nifA*-promoter region of *H. seropedicae* is very complex and contains two NtrC-, three NifA-, one IHF- (integration host factor), and a σ^{54}-binding site (Souza *et al.,* 1991; 2000a; Wassem *et al.,* 2000). Regulation of this promoter is similar to that of *K. pneumoniae* by being repressed by NH_4^+, but not by O_2, and positively activated by NtrC and dependent on σ^{54} (Souza *et al*., 2000a). Transcription of the *nifA* promoter that is activated by NtrC *in vivo* and *in vitro* is stimulated by IHF, which, in turn, impairs activation by NifA. IHF has therefore a dual role in the expression of the *nifA* promoter of *H. seropedicae*; it acts positively on the NtrC-dependent and negatively on the NifA-dependent activation (Wassem *et al.,* 2000).

6.3. Domain Structure of the NifA Proteins

The NifA protein is a transcriptional activator of σ^{54} promoters, regulating the expression of a variety of genes that include *nif* and *fix*, of most diazotrophic Proteobacteria (see Fischer 1994 and Arcondéguy *et al.,* 2001 for reviews).

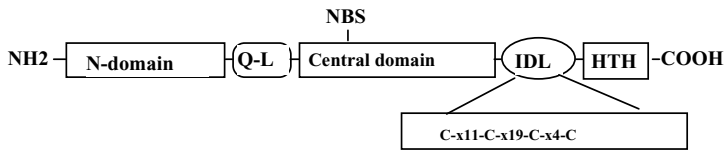

Figure 4. Schematic representation of the NifA protein structure.
QL and IDL, interdomain linkers; HTH, helix-turn-helix motif; the conserved cysteine motif found in O_2-labile NifA is schematized below the IDL motif.

NifA has three main domains (Figure 4); the N-terminal domain, the central σ^{54}-interacting catalytic domain, and the C-terminal DNA binding domain, separated by two interdomain linkers (Drummond *et al.,* 1986; see Fischer, 1994 and references therein). The N-terminal domain of NifA is quite variable in size and amino-acid composition with apparently no conserved structural pattern. The central domain is linked to the N-terminal domain by the QL linker and to the C-terminal domain by the IDL linker. The inter domain linker (IDL) also varies in size and has low homology among NifA proteins, being longest in the α– and β–Proteobacteria.

The central domain has ATP-binding consensus sequences, it contacts the RNA polymerase-σ^{54} holoenzyme, and is essential for NifA catalysis of the conversion of closed to open transcriptional complex, a process requiring ATP hydrolysis (see Buck *et al.,* 2000, for a review). Control of NifA activity is modulated by ammonia and O_2 *via* two different mechanisms, depending on its structure. In the NifA of α– and β–Proteobacteria, a conserved characteristic four-cysteines motif (C-X_{11}-C-X_{19}-C-X_4-C) spans the central domain and the IDL linker. This motif is lacking in the NifA of γ–Proteobacteria, such as *K. pneumoniae*, *A. vinelandii*, and *P. stutzeri*, and in the β–Proteobacterium, *Azoarcus* BH72 (Egener *et al.,* 2002; Desnoues *et al*., 2003)

6.4. Regulation of NifA Activity

6.4. 1. Control of NifA Activity in γ–Proteobacteria

A regulatory role for the N-terminal domain of NifA proteins was first proposed for *K. pneumoniae* by Drummond *et al.* (1990), who suggested that the role of the N-terminal domain is to block the action of NifL under derepressing conditions. Evidence suggests that the N-terminal domain of *A. vinelandii* NifA strongly influences complex formation between NifL and NifA (Money *et al.,* 1999).

In the γ–Proteobacteria, the activity of NifA is controlled by the redox flavoprotein, NifL, in response to NH_4^+ and O_2 (Dixon, 1998). Under conditions of high NH_4^+ and O_2, NifL is complexed with NifA and maintains it in a transcriptionally inactive form. Relief of NifL inactivation of NifA under NH_4^+ limitation requires the PII paralogue, GlnK (see Arcondéguy *et al.,* 2001 for a review). Under conditions of high NH_4^+ concentation, NifL forms a tight complex with NifA, which is not relieved by GlnK. NifL is also capable of sensing high O_2 concentration and, in its oxidized form, complexes to and inactivates NifA (Dixon, 1998).

An identical mechanism involving NifL, modulated by NH_4^+ and O_2, has been shown to operate in *Azoarcus* BH72 (Egener *et al.,* 2001), where three PII-like proteins (GlnB, GlnK and GlnY) are present. Neither GlnB nor GlnK was essential for nitrogen fixation and GlnY, which could not be detected in the wild-type strain BH72 but is expressed in the double mutant *glnBK*, was suggested to be sufficient to allow nitrogen fixation (Martin *et al.,* 2000). Further analysis of single and double GlnB/GlnK mutants revealed that expression of the *nifH* promoter, hence the activity of NifA, was not affected (Martin and Reinhold-Hurek, 2002).

6.4.2 Control of NifA Activity in α– and β–Proteobacteria: Role of the Cysteine Motif.

In the α– and β–Proteobacteria, where there is no NifL, NifA proteins appear to respond directly to NH_4^+ and O_2 with the N-terminal and the IDL linker plus the central domain, respectively, being involved (Fischer, 1994; Arsène *et al.,* 1996; Souza *et al.,* 1999; Arcondéguy *et al.,* 2001).

The four-cysteine motif is characteristic of the NifA proteins of the rhizobia (Fischer, 1994) and other α–Proteobacteria, such as *A. brasilense* (Liang *et al.,* 1991), *A. lipoferum* (Shigematsu *et al.,* 1997), and *G. diazotrophicus* (Lee *et al.,* 2000; Accession number AF030414) and of the β–Proteobacterium, *H. seropedicae* (Souza *et al.,* 1991; 1999). It has been suggested that this motif is directly involved in the O_2-sensitivity of these NifA proteins (Fischer *et al*, 1994; Souza *et al.,* 1999).

6.4.3. Control of NifA Activity in α– and β–Proteobacteria: *Function of the N-Terminal Domain of NifA*

The N-terminal domain is not essential for NifA activity (see Fischer, 1994; Arsène *et al.,* 1996 and references therein). A regulatory role for the amino-terminal region of NifA is well documented for those of *A. brasilense* (Arsène *et al.,* 1996) and *H. seropedicae* (Souza *et al.,* 1999), where the N-terminal domain inhibits the catalytic activity of the central and the DNA-binding activity of the C-terminal domain (Monteiro *et al.,* 1999a; 2001). The N-terminal domain of the NifA proteins of *A. brasilense* and *H. seropedicae* are involved in controlling the catalytic activity (and probably the interaction with the RNA polymerase-σ^{54} holoenzyme) in a response triggered by NH_4^+. Removal of the N-terminal domain (N-truncated NifA) led to insensitivity to NH_4^+ with no alteration in O_2 sensitivity (Arsène *et al.,* 1996, Souza *et al.,* 1999).

Evidence for the involvement of the N-terminal domain in the control of the NifA protein of *H. seropedicae* by ammonia came from studies in an *E. coli* background (Souza *et al.,* 1995). In *E. coli*, the native NifA protein is inactive, whereas an N-truncated form is active. The latter can activate a *K. pneumoniae nifH-lacZ* promoter fusion, both in the presence or absence of NH_4^+ under low O_2 tensions. O_2 had an inhibitory effect on the activity of both NifA forms. Similar experiments carried out in a *nifA* mutant of *A. brasilense* (FP10) showed that native NifA was only active under limiting NH_4^+ and O_2, whereas the N-truncated NifA was active in the presence of NH_4^+ and, as long as O_2 was limiting, both proteins were active. The results suggested that the N-terminal domain was involved in NH_4^+ sensing and also that a factor, probably capable of interacting with the N-terminal domain, was absent in *E. coli*. Under identical conditions of NH_4^+ and O_2, and in the absence of NifL, the NifA protein of *K. pneumoniae* was fully active (Souza *et al.,* 1999), thus intrinsically insensitive to NH_4^+ and O_2.

Working with a NifA protein lacking its N-terminal domain (N-truncated NifA), Monteiro *et al.* (2003) showed that NifA was inactive in an *E. coli* Fnr background, suggesting post-translational modification of NifA by a system dependent on the Fnr. This observation is reminiscent of that of *K. pneumoniae* (Grabbe *et al*., 2001), where *fnr* null mutants of *E. coli* failed to release the inhibition of *K. pneumoniae*

NifA by NifL in the absence of O_2. The iron requirement for NifA activity in *H. seropedicae* (Souza *et al.,* 1999) points to the need for a Fe-insertion pathway into NifA, possibly involving Fnr. Alternatively, or in addition, Fnr might be necessary to maintain NifA in a reduced state, again involving Fe.

6.4.4. Control of NifA Activity in α– and β–Proteobacteria: *Role of GlnB (PII)*

Several lines of evidence support the involvement of GlnB (PII) in the control of NifA activity in *A. brasilense* and *H. seropedicae*. First, an *ntrC* mutant of *A. brasilense* was Nif$^-$ (strain FP9; Pedrosa and Yates, 1984) and showed low levels of expression of the *glnB* promoter (de Zamaroczy *et al.,* 1993). Second, *glnB* mutants of *A. brasilense* (De Zamaroczy *et al.,* 1993) and *H. seropedicae* (Benelli *et al.,* 1997) are Nif$^-$. *A. brasilense glnB* mutants expressed the *nifA* gene under all conditions tested but failed to activate a *nifH-lacZ* fusion (Arsène *et al.,* 1996; Souza *et al.,* 1999). The *A. brasilense glnB* mutant was complemented for nitrogen fixation either by the *nifA* gene of *K. pneumoniae*, which is insensitive to NH_4^+ and O_2 and expressed constitutively, or by the N-truncated NifA proteins, but not by the native NifA proteins (Arsène *et al.,* 1996). Third, *in vivo* studies showed that, when the N-terminal domain of *H. seropedicae* NifA was co-expressed *in trans* at a high level in *E. coli*, the N-truncated NifA was inactive (Monteiro *et al.,* 1999b), thus behaving like the native NifA (Souza *et al.,* 1999). *In vitro* studies confirmed that the *H. seropedicae* N-terminal terminal domain is capable of physically interacting with the N-truncated protein, protecting it from proteolysis, and causing inhibition of ATPase and DNA-binding activities; thus, cross-talk between the N-terminal and the central plus C-terminal domains was proposed (Monteiro *et al.,* 2001). Fourth, replacement of a single Tyr residue at position 18 of the N-terminal domain of NifA of *A. brasilense* produced a transcriptionally active NifA that was PII independent, suggesting that the PII protein interacts specifically with the N-terminal domain of NifA (Arsène *et al.,* 1999). Fifth, interaction between PII and the NifA N-terminal domain was found using the yeast two-hybrid technique (Du *et al*., 2002).

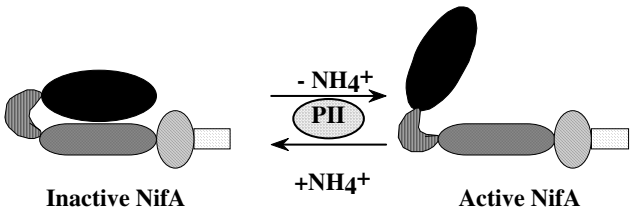

Figure 5. Model of regulation of NifA activity in A. brasilense*.*
NifA protein is active only in the absence of ammonia. The N-terminal domain is in black. The PII protein is required to prevent the N-terminal inhibition. In the presence of ammonia, the N-terminal domain inhibits the rest of the protein, probably because PII is unable to maintain NifA in its active form (adapted from Arsène et al., *1996).*

The N-terminal domain of the α-Proteobacterium, *A. brasilense*, and the β–Proteobacteria, *H. seropedicae*, have therefore a fundamental role in controlling NifA activity in response to NH_4^+ and transduced by PII/PII-UMP. A model of regulation of NifA activity has been proposed (Figure 5) for this multiple interaction mechanism in *A. brasilense* (Arsène *et al.,* 1996). Accordingly, under NH_4^+-limitation, PII, probably in the PII-UMP form, relaxes NifA by unfolding the N-terminal-QL bend, exposing the central and C-terminal domains, so rendering NifA active for ATP-dependent catalysis and binding to DNA. Under conditions of high NH_4^+, PII, probably in the non-uridylylated form, promotes or is unable to interact with NifA, allowing the N-terminal domain to loop over QL and contact the central and C-terminal domains, so rendering them inactive. The recent data presented for *H. seropedicae* NifA supports an apparently identical model.

7. CONCLUSIONS

An increasing number of nitrogen-fixing species associated with grasses and other cash crops, such as sugarcane and coffee, are being regularly discovered. In most cases, we do not have much information on their *nif* genes except for phylogenetic studies based on conservation of the nitrogenase structural-genes sequences (see Chapter 2, 9, 10, and 13). A striking example is *Burkholderia.* Non-cultivable endo-cellular bacteria, related to *Burkholderia* and living in symbiosis with arbuscular mycorrhizal fungi, have been described (Bianciotto and Bonfante, 2002). Several novel species have been discovered, often characterized as endophytes, for which there little knowledge on *nif* genes (Marin *et al.*, 2003). Availability of genomics projects, such as that of *H. seropediacae*, open new perspectives for further developments of *nif* genetics of newly discovered species.

A substantial amount of information on the regulatory cascades that control nitrogen fixation and assimilation in a few species is reported here. In particular, the involvement of PII and parologues in the regulatory cascades remain of major interest. These should reflect different properties both at the free-living and associated state. Yet, little information is available on the expression of *nif* and associated genes within the plant. Further knowledge of the involvement of GlnK in the regulation of NH_4^+ transport and in the multiplicity of mechanisms that control both nitrogenase and NifA activities is relevant if we are to understand what controls and what limits nitrogen fixation in plant tissues. This information would help to evaluate the potential role of nitrogen fixers associated with grasses and to engineer better nitrogen-fixing associations.

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Chapter 4

CHEMOTAXIS IN SOIL DIAZOTROPHS: SURVIVAL AND ADAPTATIVE RESPONSE

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1. INTRODUCTION

Soil is a heterogeneous environment and niches for microorganisms include soil pores, soil aggregates, and root surfaces (rhizosphere). Soil bacteria are subjected to considerable seasonal fluctuations in environmental conditions. Root exudates and rhizodeposition form the main source of nutrients for microorganisms in the rhizosphere and amino acids, sugars, and organic acids are considered as the major exudate compounds providing rhizospheric bacteria with essential sources of carbon and fixed-nitrogen. Even so, fixed-nitrogen is often a limiting factor in the soil. Therefore, nitrogen fixers that can find a microenvironment depleted of O_2, which is harmful for nitrogenase, will have a competitive advantage. In addition, respiration by the roots, together with the metabolic activities of the indigenous rhizospheric microflora, contributes to both the formation of O_2 gradients and to fluctuations in the redox potential in the rhizosphere. Finally, fluctuation in the water content of the soil matrix and the limited diffusion of O_2 in water may also contribute to the formation of O_2 and redox gradients in soil aggregates and soil pores.

Directed movement in response to natural gradients of different stimuli is called "taxis". Soil bacteria that can sense and actively move toward niches where conditions are optimum for growth and survival will have a competitive advantage because they could rapidly navigate to favourable niche by directed motility rather than by chance. Motile sensory behaviour is proposed to play a key role in the establishment of various plant-microbe interactions, such as associative and symbiotic relationships between plants and microorganisms, as well as in pathogenesis (Armitage *et al.*, 1988; Caetano-Anolles *et al.*, 1988a; 1988b; 1992; Dharmatilake and Bauer, 1992; Zhulin and Armitage, 1992; Yost and Hynes, 2000). Active motility may also contribute to the localized spread of bacteria within

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microenvironments, such as soil pores, provided that there is a suitable water film present to allow motility to take place (Bashan and Holguin, 1994; Turnbull *et al.*, 2001).

2. GENE-EXPRESSION REGULATION AND CHEMOTAXIS AS ADAPTIVE RESPONSES TO ENVIRONMENTAL CHANGES

As unicellular organisms, prokaryotes are constantly interacting with the environment *via* their entire bodies. Because environmental conditions fluctuate constantly and sometimes dramatically, microorganisms face a challenge to keep up with these fluctuations in order to survive. Survival strategies adopted by microorganisms include sensory and regulatory capabilities. First, a cell must recognize (sense) a physico-chemical parameter that has changed and then trigger (regulate) an adaptive response, if necessary. Physico-chemical parameters that are important to and can be recognized by microorganisms (also referred to as "environmental cues" or "stimuli") include concentrations of various chemicals (ranging from simple inorganic ions to complex organic polymers), light, temperature, redox potential, osmotic pressure, *etc*. (Miller and Wood, 1996; Taylor *et al.*, 1999).

The survival strategy that is most frequently found in prokaryotes is regulation of gene expression. Most pathways that regulate gene expression in prokaryotes consist of a sensor histidine kinase and a cognate response regulator (the so-called "two-component regulatory system"; Hoch and Shilavy, 1995). A sensor histidine kinase recognizes a stimulus, when it interacts with the sensory domain of the kinase, and then transmits the information in the form of a phosphoryl group to the partner protein, a response regulator. Upon phosphorylation, the response regulator binds to specific promoter sites and affects transcription of a particular set of genes. Most response regulators of the two-component systems act as transcriptional regulators, however, newly emerging data suggest that two-component systems can also directly regulate the enzyme activity in response to changes in the environment. Therefore, there seems to be slow (regulation of gene expression) and fast (regulation of enzyme activity) adaptive responses that are parts of an overall survival strategy of prokaryotes. These adaptive responses are found throughout the prokaryotic world and genomics studies have identified two-component systems or similar regulatory pathways in all microorganisms except for a few intracellular pathogens that have lost most of their genome content due to their parasitic lifestyle.

In addition to these basic survival strategies, many (if not most) microorganisms have adopted what we can call a super-fast response to changes in the environment; the regulation of cell motility. This strategy is simple and efficient and is designed to quickly relocate the entire organism into the microenvironment that is the best for its growth and survival. There is a pertinent parallel with the eukaryotic world, where most organisms, including humans, but not all (*e.g*., plants) rely heavily on their ability to move toward favourable and away from harmful conditions. Active movement of microorganisms toward (or away from) different physico-chemical parameters is collectively known as chemotaxis (Aizawa *et al.*, 2002).

3. MOLECULAR MECHANISM OF THE CHEMOTACTIC RESPONSE: LEARNING FROM *ESCHERICHIA COLI.*

A molecular mechanism of chemotaxis has been elucidated using the model organisms, *Escherichia coli* and *Salmonella typhimurium* (Bren and Eisenbach, 2000), and to some degree, *Bacillus subtilis* (Aizawa *et al.*, 2002), but not any of the nitrogen-fixing plant-associated species. The data on chemotaxis obtained with *B. subtilis* and an archaeon, *Halobacterium salinarum* (Hou *et al*., 2000; Kokoeva and Oesterhelt, 2000; Aizawa *et al*., 2002), were even considered as artefacts until recent studies on a variety of other species and the arrival of genomics uncovered the true diversity of the chemotaxis system. Still, a good understanding of the molecular design of the chemotactic response is available only for *E. coli*.

3.1. Motility

E. coli exhibits a random swimming pattern of straight smooth swims interrupted by directional changes brought about by a transient reversal in the rotation of the flagellar motor (Figure 1). This causes the cell to tumble and then change the direction of swimming. In a positive environment of increasing attractant or decreasing repellent, *E. coli* cells suppress directional changes, which results in an increase in the duration of smooth swimming (Bren and Eisenbach, 2000). In this manner, cells move to an optimal environment.

3.2. Chemoreceptors

Changes in various physico-chemical parameters, such as nutrients, salts, O_2 and alternative electron acceptors, light, and temperature, are detected by specialized chemoreceptors, also known as methyl-accepting chemotaxis proteins (MCPs). *E. coli* has five chemoreceptors; four trans-membrane methyl-accepting chemotaxis proteins (Tsr, Tar, Trg and Tap) and one membrane-associated cytoplasmic receptor Aer (Rebbapragada *et al.,* 1997). The mechanism of trans-membrane signalling by *E. coli* chemoreceptors has been studied in great detail (Falke and Hazelbauer, 2001) and a crystal structure is available for both the Tsr and Tar chemoreceptors (Peach *et al.,* 2002; Weis *et al.,* 2003). Binding of an attractant or a repellent to a periplasmic N-terminal sensory domain of the receptor causes a conformational change in its cytoplasmic C-terminal signalling domain, which interacts with the cytoplasmic machinery comprising the excitation pathway (see below).

A survey of genomes revealed that trans-membrane chemoreceptors are widespread among chemotactic species (Zhulin, 2001), where a similar mechanism is expected. The mechanism of signalling in cytoplasmic receptors is not well-understood, but it is likely to involve direct interaction between the sensory and signalling domains. Several chemoreceptors have been studied in detail in *B. subtilis* and *H. salinarum* (Hou *et al.*, 2000; Kokoeva and Oesterhelt, 2000; Aizawa *et al.*, 2002).

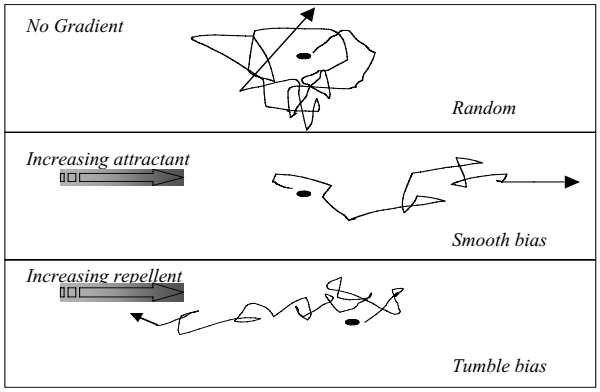

Figure 1. Schematic representation of the chemotactic behaviour of free-swimming bacteria. When a gradient of a stimulus is present, the motility of the cells is biased. See text for details.

3.3. Excitation and Adaptation Pathways

A conformational change in a chemoreceptor affects its interaction with the central regulator of chemotaxis, the CheA protein. CheA is a histidine kinase that autophosphorylates. CheA is docked to a chemoreceptor by an auxiliary protein CheW. Phosphorylated CheA serves as a phosphodonor for the cognate response regulator, CheY, and the methyltransferase, CheB (Figure 2). Phosphorylated CheY interacts with the flagellar motors responsible for swimming motility and causes a directional change. CheZ is a phosphatase that dephosphorylates CheY. Because the CheY/motor interaction is transient, the motor returns to its pre-stimulus rotation mode and the cells swims smoothly again. The cascade of the chemoreceptor-CheW-CheA-CheY-flagellar motor is known as an adaptation pathway.

Adaptation is an essential property of any sensory system, including bacterial chemotaxis. In *E. coli*, sensory adaptation of chemotaxis is mediated by covalent modification of chemoreceptors by methylation and demethylation. CheR is a methyltransferase, which constitutively methylates specific glutamyl residues of the chemoreceptor cytoplasmic domain (Bren and Eisenbach, 2000). Addition of methyl groups causes the chemoreceptor to "reset", which initiates CheA autophosphorylation, regardless of the status of the sensory domain. This ultimately causes the bacteria to change swimming pattern from smooth to random. In this way, cells are able to swim to and remain in positive environments. CheA controls the activity of the CheB methylesterase. CheB removes methyl groups (that were

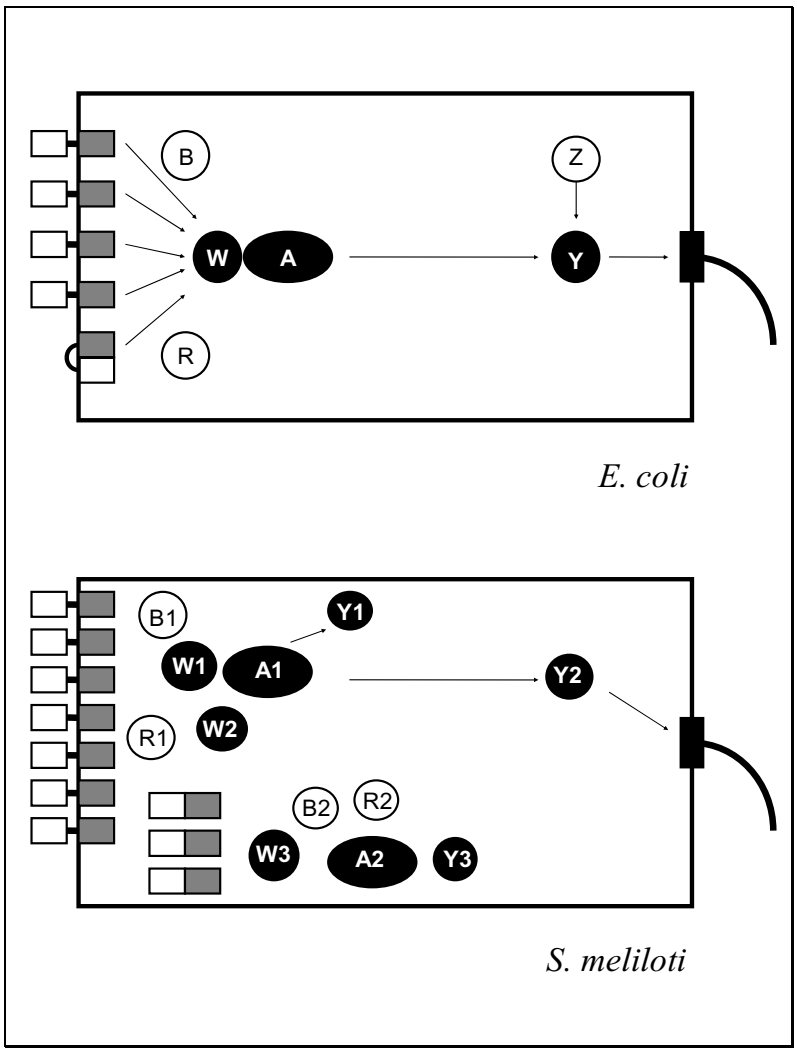

Figure 2. Schematic representation of the chemotaxis machinery in E. coli *and* S. meliloti*. Chemoreceptors are shown as a combination of a sensory (white rectangle) and signalling (grey rectangle) domain. Multiple homologs of* E. coli *chemotaxis proteins (Che A, B, R, W, Y, Z) are present in* S. meliloti*. See text for details.*

added by CheR) from the chemoreceptors, so allowing receptors to once again become sensitive to changes in the environment. Thus, CheR and CheB comprise the adaptation pathway in chemotaxis.

4. DIRECTED MOTILITY IN SOIL DIAZOTROPHS

4.1. Sinorhizobium meliloti

Rhizobia comprise a diverse group of organisms and different types of motility are found in different rhizobial species. So far, chemotaxis is best studied in *Sinorhizobium meliloti* (Armitage and Schmitt, 1997; Scharf and Schmitt, 2002). This bacterium possesses a relatively rare type of motility through which the cells swim forward by means of a unidirectional intermittent rotation of a single flagellum (Gotz and Schmitt, 1987). However, this unusual motility is regulated by a conventional chemotaxis system. As *E. coli*, *S. meliloti* possesses a CheA/CheY two-component system that transmits a signal from an array of chemoreceptors to the flagellar motor. Binding of the phosphorylated CheY to a switch protein on the motor causes a stop, in contrast to a change in the direction of rotation in *E. coli*. Although the CheA/CheY system of *S. meliloti* is homologous to that of *E. coli* and performs essentially the same function, there is a significant variation in the overall design of the molecular chemotaxis machinery (Figure 2).

First, the number of chemoreceptors in *S. meliloti* is twice as high as in *E. coli*. Furthermore, in addition to membrane-bound receptors, there are soluble cytoplasmic receptors in *S. meliloti*. Stimuli that are recognized by these multiple chemoreceptors are currently unknown, however, it is likely that some of them might be of the plant origin. Second, the CheZ phosphatase, which serves as a "terminator" of the chemotactic signal in *E. coli* by dephosphorylating the CheY protein, is missing from *S. meliloti*. Dephosphorylation of motor-binding CheY (CheY2) in *S. meliloti* is partly achieved by another CheY homolog (CheY1), which serves as a "phosphate sink" (Sourjik and Schmitt, 1996; 1998). Third, there are additional homologs of chemotaxis-gene products in *S. meliloti* (Figure 2) for which function is unknown. Overall, the organization of the molecular pathway for chemotaxis in this bacterium appears to be much more complex than that in *E. coli* and its functioning remains to be thoroughly investigated. In contrast to *E. coli*, chemotaxis in *S. meliloti* is thought to be metabolism-dependent and responses to major chemo-attractants do not seem to involve methylated chemoreceptors (Robinson and Bauer, 1993). *S. meliloti* also has an unusual behavioural response to O_2; in contrast to most other species studied in this respect, *S. meliloti* shows chemokinesis, but not taxis, in O_2 gradients (Zhulin *et al.*, 1995).

4.2. Rhizobium leguminosarum

Chemotaxis in *R. leguminosarum* has been studied since the early 1980s at least. These pioneering studies focused primarily on identifying chemo-attractants. Bowra and Dilworth (1981) sought to identify both the optimum conditions for studying chemotaxis in *R. leguminosarum* and to test its response to sugars and sugar alcohols using the capillary assay. Next, Gaworzewska and Carlile (1982) used the capillary assay to test the *R. leguminosarum* response to fractions of root exudates from *Pisum sativum* and other plants as well as to purified compounds,

such as amino acids, sugars, and organic acids. These two studies identified some 37 chemo-attractants for *R. leguminosarum*.

When more information became available about the components of root exudates from plant species that induce expression of bacterial genes involved in nodulation, such as the flavone apigenin and the flavonones naringenin and hesperitin, the chemotactic response of *R. leguminosarum* to these compounds was studied (Firmin *et al.*, 1986; Armitage *et al.*, 1988). Using the chemotaxis-well assay, taxis towards the nodulation inducers, apigenin and naringenin. as well as to the nodulation anti-inducer, kaempferol, was detected. The minimum threshold concentrations for these compounds were found to be on the order of 5-50 μM, which was about one-tenth of that of other chemo-attractant compounds, such as sugars, amino acids, and weak organic acids. However, these threshold concentrations do not represent the concentrations actually sensed by the cell. Such a determination could be made only with a temporal assay, which would measure individual cellular responses. Moreover, comparisons of these studies highlight the possibility of individual strain variances, which must be considered.

When the symbiotic plasmid from *R. leguminosarum* was sequenced and analysed, Brito *et al.* (1996) reported finding a gene apparently involved in chemotaxis, specifically a chemoreceptor. However, no phenotype associated with this gene was demonstrated. Yost *et al.* (1998) were the first to report phenotypes for receptors in *R. leguminosarum*. Transposon mutants, which were defective in five genes encoding MCPs, were generated and then screened for deficiencies in chemotaxis to a suite of known chemo-attractants using the swarm-plate assay. The mutant lacking the *mcpB* gene was the only strain to show impaired chemotaxis. This mutant exhibited a reduced swarm diameter on all the compounds tested.

In addition to the swarm-plate assays, Yost *et al.* (1998) tested the ability of the mutants that lacked MCPs to successfully compete with the wild type in root-nodule formation. Pea plants were inoculated with a ~1:1 ratio of wild type to mutant for *mcpB*$^-$ and ~1.5:1 for *mcpC*$^-$. In both cases, the recovery ratio of mutant to wild type was less than 0.25:1. More recently, Yost *et al.* (2003) characterized an additional gene encoding an MCP, *mcpG*, which is found on the nodulation plasmid of *R. leguminosarum*, thereby leading to speculation that this protein might be involved in the nodulation process. However, as with previous attempts, no clear phenotype was identified.

Available genomic data indicates that *R. leguminosarum* has more than 20 chemoreceptors and two chemotaxis pathways, one of which is homologous to a major chemotaxis pathway in *S. meliloti* and the other to one of chemotaxis pathways from *Rhodobacter sphaeroides*, a nitrogen-fixing α–Proteobacterium (Miller, Alexandre, and Zhulin, unpublished observations).

4.3. Azospirillum brasilense

Although azospirilla are generally less well-studied than rhizobia, chemotaxis in those free-living diazotrophs that are associated with roots of grasses and cereals (especially *Azospirillum brasilense*) has been studied in great detail. Migration of

azospirilla in the soil and towards plants was reported previously (Bashan and Levanony, 1990). Colonization of the plant root surface by *A. brasilense* is thought to be dependent on both active motility and chemotaxis toward plant-root exudates (Zhulin and Armitage, 1992). Flagellation in azospirilla is discussed in Chapter 5 of this volume.

4.3.1. Aerotaxis

In *A. brasilense*, the strongest behavioural response is aerotaxis (taxis toward O_2), first described by Barak *et al.* (1982). Aerotaxis guides the bacteria to a preferred low O_2 concentration (4 μM), which appears optimal for both energy generation and nitrogen fixation (Zhulin *et al.*, 1996). Hence, aerotaxis is an important adaptive behavioural response that can guide free-living diazotrophs to the optimal niche for nitrogen fixation in either the soil or the rhizosphere (Zhulin *et al.*, 1996).

4.3.2. Chemotaxis

A chemotactic mutant (genetically undefined) of *A. brasilense* was impaired in root-surface colonization (Vande Broek *et al.*, 1998), suggesting that chemotaxis is critical for the establishment of *Azospirillum* in the rhizosphere of hosts plants. In azospirilla, chemotaxis toward root exudates has been described as strain-specific (Reinhold *et al.*, 1985). Strains of both *A. brasilense* and *A. lipoferum*, after isolation from C4 plants, were attracted by substances present at a high level in C4 plant-root exudates, whereas *Azospirillum* strains isolated from C3 plants were attracted by C3 plant-root exudates. This observation suggested that chemotaxis was metabolism-dependent.

Indeed, chemotaxis to most chemical attractants and to all repellents is metabolism-dependent in *A. brasilense* (Zhulin and Armitage, 1993; Alexandre *et al.*, 2000), which is in contrast to *E. coli*, where most chemicals are sensed *via* a ligand-binding metabolism-independent mechanism. Organic acids and carbohydrates are the best attractants for *A. brasilense* and, concomitantly, they are the best growth substrates (Barak *et al.*, 1983; Alexandre *et al.*, 2000). Amino acids are weak attractants for these bacteria that fix nitrogen under microaerophilic conditions (which cells find *via* aerotaxis) and, therefore, do not rely on amino acids as an external fixed-nitrogen source for growth. Again in contrast to *E. coli*, non-metabolizable analogues of metabolizable chemoattractants are not attractants and, moreover, they inhibit chemotaxis to the metabolizable analogues (Alexandre *et al.*, 2000).

The intracellular chemotaxis machinery of *A. brasilense* appears to be similar to that of other bacteria. A major chemotaxis operon, which contains homologs of chemotaxis proteins, has been identified in *A. brasilense* (Hauwaerts *et al.*, 2002). In addition to CheA, CheW and CheY, it encodes CheB and CheR, indicating that methylated chemoreceptors are present in *A. brasilense*, although it was previously suggested that chemotaxis to major attractants in this species is methylation-independent (Zhulin and Armitage, 1993). In addition, a structural gene coding for a chemoreceptor (*mcpAb*) is present within the *nif*-gene cluster of *A. brasilense* (Potrich *et al.*, 2001).

In the presence of root exudates, *A. brasilense* produces the SbpA protein, a homolog of the ChvE protein of *A. tumefaciens*, which is involved in transport and chemotaxis to certain sugars. The *sbpA* mutant was impaired in the transport of sugars, such as D-galactose, and in the chemotactic response to these compounds (Van Bastelaere 1999; see also Chapter 5 of this volume).

4.3.3. Energy Taxis as the Main Mechanism for Directed Motility.

A functional electron-transport system is required not only for aerotaxis, but also for chemical sensing, and a terminal cytochrome oxidase is required for a full-scale aerotactic and a chemotactic response to all major attractants (Alexandre *et al.*, 2000). The mutant strain that lacks a component of the *cbb3* cytochrome oxidase was impaired in aerotaxis, redox taxis, and chemotaxis (Alexandre *et al.*, 2000). This suggests that chemotaxis in *A. brasilense* occurs *via* the energy-taxis mechanism (Alexandre *et al.*, 2000; Alexandre and Zhulin, 2001).

The signal for this type of behaviour originates in the electron-transport system, where a change in the rate of the electron transport (or a related parameter) is detected by specialized chemoreceptors and transmitted to flagellar motors *via* the chemotaxis-excitation pathway (Taylor and Zhulin, 1998; Alexandre and Zhulin, 2001). Chemicals that interfere directly with the electron-transport system cause a repellent response in *A. brasilense* (Alexandre *et al.*, 2000). Adaptation of the quantitative temporal-gradient assay to study chemotaxis in *A. brasilense* was critical to characterizing chemotaxis in this organism (Alexandre *et al.*, 2000) and allowed the detection of subtle differences in behavioural patterns.

4.4. Other Species

Many soil diazotrophs are motile and chemotactic. Interestingly, the *mcpA* gene that codes for a chemoreceptor was also found within the *nif* cluster of *Gluconacetobacter diazotrophicus* (Lee et al; 2001, see also Chapter 10 of this volume), similar to the situation in *A. brasilense* (see above). The role of behaviour in the ecology of soil diazotrophs remains largely unknown due to a lack of understanding of the mechanism of their motility and chemotaxis.

Advances in microbial genomics, however, provide the first insights into the organization of the chemotaxis machinery in some of these species. For example, a preliminary analysis of the complete genome sequence of *Mezorhizobium loti* indicated that this organism lacks flagellar motility, which is typical of *S. meliloti* and *R. Leguminosarum*. However, *M. loti* appears to have motility based on the type IV pili, which is regulated by a chemotaxis-like signal-transduction cascade (I. B. Zhulin, unpublished observations). The nearly complete genome sequence of another soil diazotroph, *Azotobacter vinelandii*, allowed the identification of its flagellar and chemotaxis systems, which are homologous to those of *E. coli* (I.B. Zhulin, unpublished observations). Interestingly, the *A. vinelandii* genome contains more chemoreceptors than the *E. coli* genome. The trend toward large sets of chemoreceptors is clearly observed in soil bacteria and may represent the necessity for these organisms to detect diverse stimuli typical of their environments.

5. FUTURE STUDIES

Emerging genomic and experimental evidence strongly suggests that the chemotaxis machinery in microbial species, which inhabit complex environments (including soil and the rhizosphere), is significantly more complex than that in the model organism for chemotaxis, *E. coli*. Therefore, future studies should be focused on understanding both the molecular organisation of chemotaxis signal-transduction pathways in soil diazotrophs and their role in plant-microbe interactions. Specifically, the role of multiple homologs of chemotaxis proteins in different species should be addressed. It is important to stress that quantitative assays similar to those developed for studying *E. coli* chemotaxis should be adopted for studying chemotaxis in other species. Chemotaxis is a strategy used by microorganisms in order to adapt to their environment (soil, hosts etc.), therefore, advances in the understanding of the mechanisms of chemotaxis will ultimately benefit the field of biofertilizer design and bioremediation.

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Chapter 5

MOLECULAR GENETICS OF RHIZOSPHERE AND PLANT-ROOT COLONIZATION

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1. INTRODUCTION

Root colonization is a key factor in the successful interaction of plant growth-promoting rhizobacteria with plants. In order to achieve an efficient colonization, several properties are required of the bacterial. Firstly, bacterial motility is a prerequisite to respond chemotactically and to move towards plant roots, where they can benefit from root exudates as a carbon and energy source. In general, the rhizosphere is considered as that part of the soil where bacteria are under the influence of the plant root. Motility also renders them competitive with other microorganisms for the most favourable niches. Once in the vicinity of the root, attachment to target cells on the plant surface can occur. The attachment of the bacteria to plant roots is a second requirement for the establishment of a bacteria-root association. Finally, bacteria must then dispose of features that allow them to survive under variable conditions and which are thought to promote their competitiveness in the rhizosphere.

In this chapter, we give an overview of the mechanisms by which associative diazotrophic bacteria establish themselves in the rhizosphere and colonize the root system. This overview focuses in particular on the molecular genetics of: (i) bacterial motility; (ii) attachment to the root surface; and (iii) rhizosphere competence. Chemotaxis, an important feature in bacterial motility towards roots, is reviewed in Chapter 4 of this volume.

The best-known root surface-colonizing diazotrophic bacteria are represented by the genus *Azospirillum*. Other diazotrophic bacteria, such as *Azotobacter* and *Klebsiella*, are well studied in terms of their nitrogen-fixation capabililty, but are less well defined as root colonizers. Diazotrophic bacteria, named endophytes, infect the interior of plant tissues without causing symptoms of plant disease.

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Examples of such endophytes are *Azoarcus* sp., *Herbaspirillum seropedicae*, *Gluconacetobacter diazotrophicus* (reviewed in Reinhold-Hurek and Hurek, 1998), *K. pneumoniae* 342 (Chelius and Triplett, 2000), *Pseudomonas stutzeri* A15 (Rediers *et al*., 2003), and *Pantoea agglomerans*. Little is known about the way these endophytes colonize roots and enter plant tissues. Studies concerning the establishment of their endophytic nature are broadly discussed in chapter 9, 10 and 13 of this volume. As several reviews report, there is an enormous biodiversity of nitrogen fixers in the rhizosphere of plants (Döbereiner and Pedrosa, 1987; Elmerich *et al*., 1992) and the list of organisms discussed herein is not exclusive. Moreover, the distinction among bacteria as root colonizers, saprophytes or endophytes is often unclear and disputable.

2. MOTILITY OF ASSOCIATIVE DIAZOTROPHS

Motility is needed both to reach the most favourable niche and to compete with other microorganisms for those niches. It can be achieved by different mechanisms. The majority of motile prokaryotes move by means of flagella, but spirochetes are motile by means of axial filaments (also called axial fibrils or axial flagella). These axial filaments do not extend from the bacterial surface but are wrapped around the cell in the periplasmic space. By contracting these filaments, the bacteria display a corkscrew-like movement. Other bacteria, like *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, move on a solid surface by a process called twitching motility, involving short discontinuous movements of up to several micrometers (McBride, 2001). Certain cyanobacteria and myxobacteria can move over surfaces by gliding motility, a smooth translocation of cells that does not require flagella. The exact mechanism remains to be elucidated but different types of gliding mechanisms appear to be present (McBride, 2001). For grass-associated diazotrophs, motility is essentially linked to the presence of flagella and this has been particularly well studied in *Azospirillum* species.

2.1. Flagella Types

Flagella are long thin appendages, consisting of a long helical filament, which is driven by a rotary motor that is anchored in the cytoplasmic membrane and the cell wall (Macnab, 1996). The major component of the flagellar filament is a protein called flagellin. *A. brasilense*, *A. lipoferum*, and *A. irakense* display a mixed flagellation pattern (Tarrand *et al.*, 1978; Khammas *et al.,* 1989). They possess a single polar flagellum, when grown in liquid media, and several additional lateral flagella of smaller size and shorter wavelength, when grown on solid media (Figure 1). The two types of flagella of *A. brasilense* are antigenically different (Hall and Krieg, 1984). Both *A. halopraeferens* and *A. amazonense* display only the polar flagellum (Magelhães *et al.,* 1983; Reinhold *et al.,* 1987; Falk *et al.,* 2002). *Pseudomonas* spp., *Azoarcus* sp. strain BH72, and *Herbaspirillum seropedicae* have polar flagella, whereas *Azotobacter chroococcum*, *A. vinelandii*, and *A. paspali* have

peritrichous flagella (Sadoff, 1975; Lifshitz *et al.,* 1986; Döbereiner and Pedrosa, 1987).

Several bacterial species, including *Azospirillum brasilense*, can adapt their flagellation pattern in response to the environmental conditions they encounter. Swarming-cell differentiation is a remarkable example of this phenomenon. It has been extensively studied in *Proteus mirabilis* (Fraser and Hughes, 1999). Growth on surfaces coincides with cell elongation and induction of high numbers of flagella. These long, hyperflagellated cells (called 'swarm cells') are able to move over the surface in a process called 'swarming'. In *Azospirillum*, the swimming of a *laf1*::Km mutant, devoid of lateral flagella, was not different from wild-type swimming behaviour (Moens *et al*., 1995a). However, spreading over the surface ('swarming') occurred on 0.6% agar only for the wild-type Sp7 strain and not for the mutant that was devoid of lateral flagella. This observation indicates that the swarming of *A. brasilense* is a well-defined phenomenon that occurs on the surface of swarm plates solidified with 0.6% agar and that depends on the presence of lateral flagella (Moens *et al*., 1995a).

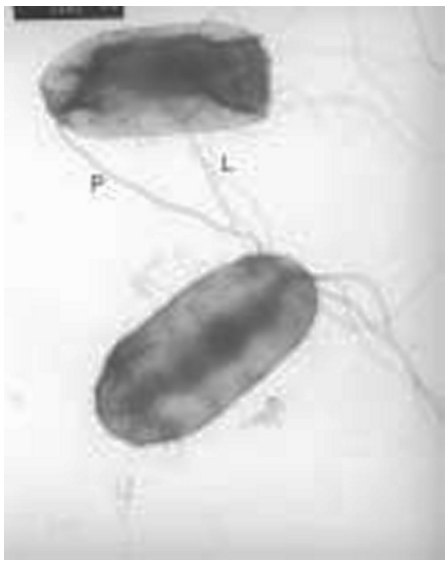

Figure1. Transmission electron micrograph of negatively-stained A. brasilense *Sp7 from a solid medium with single polar (P) and several lateral (L) flagella.*

Apparently, in *Azospirillum,* the functioning of the polar flagellum and induction of lateral flagella are somehow coupled. Moens *et al.* (1996) demonstrated that hindrance of rotation of the polar flagellum, either by growing cells in high-viscosity medium or by adding an antipolar flagellin-specific antibody to liquid

grown cells, induces expression of the structural gene of the lateral flagellin. This mechanism of coupling, however, remains to be elucidated.

2.2. Genetics of Motility

The structural gene that codes for the flagellin of the lateral flagella has been isolated in *A. brasilense* Sp7 (Moens *et al.,* 1995a). The gene, designated as *laf1*, has considerable homology with the *flaABC* genes of *Agrobacterium tumefaciens* and the *flaAB* genes of *Sinorhizobium meliloti* (Pleier and Schmitt, 1991). In *S. meliloti*, the *flaAB* genes code for different subunits of the flagellin, having a different function in the assembly of the flagella. In *A. brasilense*, there appears to be only one subunit type.

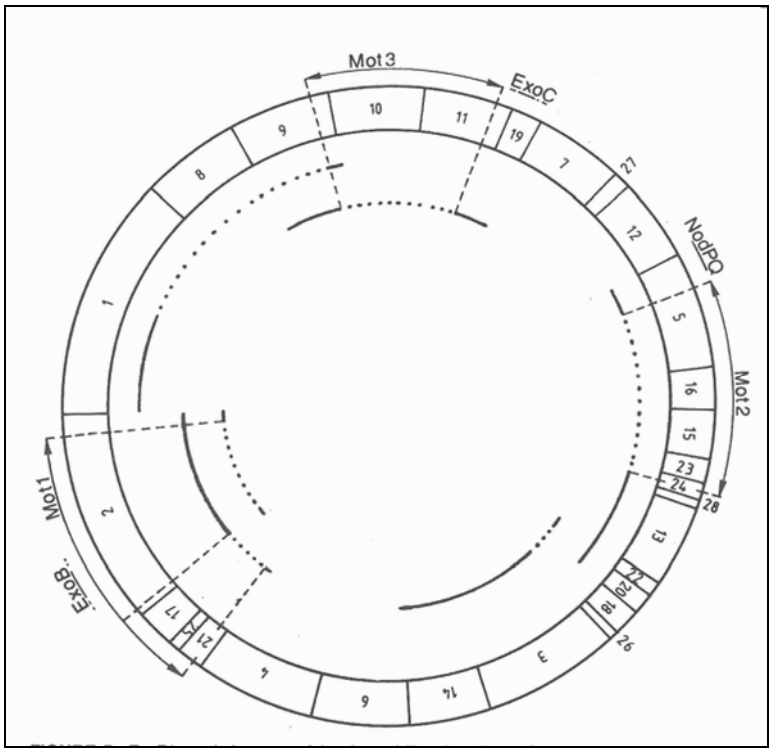

Figure 2. EcoR*I restriction map of the* A. brasilense *Sp7 90-MDa plasmid. Lines inside of the circular map represent the recombinant cosmids that were used in the construction of specific deletions. The dotted part of the line corresponds to the deleted DNA, Mot1, Mot2, Mot3 are loci involved in motility;* exoB, exoC *are loci complementing* S. meliloti exoB *and* exoC *mutants, respectively;* NodPQ *are loci homologous to the* S. meliloti nodPQ *genes (Adapted from Croes* et al.*, 1991).*

The structural gene that encodes the flagellin of the polar flagellum, Fla1, has not yet been isolated. Structural analysis of the polar flagellum demonstrated that the major protein is glycosylated (Moens *et al.*, 1995b), based on specific carbohydrate staining of protein gels, the effect of glycosylation inhibitors, chemical deglycosylation, and the use of sugar-specific monoclonal antibodies. Moreover, mutations that affect the synthesis of specific sugar nucleotides in *A. brasilense* also change the size of the polar flagellin on a denaturing polyacrylamide gel. *A. brasilense* is the first bacterium to be reported with a glycosylated flagellin protein.

Milcamps *et al.* (1996) demonstrated that an *A. brasilense ntrA* (*rpoN*) mutant is non-motile and devoid of both the polar and the lateral flagella. NtrA control of flagella synthesis was also reported for *P. aeruginosa* and *P. putida*, *Caulobacter crescentus*, and *Campylobacter coli*.

Loci governing motility in *A. brasilense* were found to be located on the chromosome as well as on the 90-MDa plasmid (Figure 2) (van Rhijn *et al.,* 1990; Croes *et al*., 1991). Deletion analysis of the *A. brasilense* Sp7 90-MDa plasmid revealed the presence of three regions with an impact on motility (Mot loci) (Croes *et al.,* 1991). Two loci, Mot1 and Mot2, are likely to be involved in either the synthesis or functioning of the lateral flagella because deletion mutants in Mot1 and Mot2 displayed an altered motility on solidified medium (swarming) but not in liquid medium (swimming). A deletion mutant in the third locus, Mot3, was shown to be completely non-motile in both liquid medium and on solid surfaces and to lack both the polar and lateral flagella. Hence, locus Mot3 might have either a regulatory function in the production of the flagella or could encode an essential structural component for both types of flagella.

2.3. Motility and Root-Colonization Efficiency

In natural environments, soil moisture is the limiting factor in migration of *Azospirillum brasilense* towards plant roots (Bashan, 1986). This suggests that free swimming plays a major role in motility in natural environments. In general, *A. brasilense* is pushed forward by a counter clockwise-rotating polar flagellum with occasional brief periods of clockwise-rotation causing reversals that serve to reorient the cell (Zhulin and Armitage, 1993). The capacity of *A. brasilense* to initiate wheat-root colonization was investigated with different mutant strains equipped with a constitutively expressed *gusA* reporter gene. Only non-flagellated mutants and a non-chemotactic mutant exhibited strongly reduced colonization ability. This demonstrated directly the requirement of bacterial motility for initiation of wheat-root colonization (Vande Broek *et al.,* 1998).

Two strains of *A. lipoferum*, a motile laccase-negative strain (4B) and a non-motile laccase-positive strain (4T), have been isolated simultaneously and at the same frequency from the rice rhizosphere. Both strains are able to colonize rice roots efficiently, but the motile form 4B remains dominant. Therefore, *A. lipoferum* 4B is likely the better colonizer of the rice rhizosphere compared to strain 4T and this may be, in part, due to its motility (Alexandre *et al.,* 1996). Moreover, after inoculation of rice roots with *A. lipoferum* 4B, spontaneous stable non-motile

laccase-positive forms (4T) appeared in high numbers. *In vitro* evidence suggests that the non-motile laccase-positive *A. lipoferum* strain emerged from the motile laccase-negative wild-type strain (4B) *via* a two-step phenotypic switching event. The initial generation of an intermediate non-motile laccase-negative form from the wild type *via* a phase variation-like process is then followed by the appearance of a non-motile laccase-positive variant under extremely low O_2 concentrations. By this process, bacteria may adapt to near anaerobic conditions typical of the rice rhizosphere (Alexandre and Bally, 1999).

3. ATTACHMENT TO PLANT ROOTS

Attachment of bacteria to plant roots is essential for efficient association with the host plant. It anchors the bacteria to the roots for better access to plant exudates and it prevents bacteria from being washed away. On the other hand, attachment of beneficial bacteria protects the plant against attacks of non-beneficial colonizers. Moreover, substances excreted by bacteria reach the plant before consumption by other bacteria. Although the involvement of extracellular polysaccharides and proteins are indicated in the root-attachment process, the precise attachment mechanism remains unclear. The difficulty in elucidating this process is due to the variety of bacterial surface components involved and the high number of factors affecting this adhesion process, such as culture growth conditions, and the physical and chemical conditions of the binding assay.

Several bacterial surface structures have been identified as being involved in the attachment process. In addition to their locomotive properties, adhesive properties have been attributed to bacterial flagella. Further, other adhesins, such as pili, polysaccharides or outer membrane proteins, seem to be involved.

3.1 Learning from Heterologous Systems

In order to isolate *Azospirillum* genes involved in plant interaction and owing to the absence of an easily detectable phenotype, putative *Azospirillum*-plant association genes were initially isolated on the basis of DNA-sequence homology. In this way, sequences homologous either to essential genes for attachment and tumour formation of plant cells in *A. tumefaciens* (Waelkens *et al.,* 1987; Raina *et al.,* 1995) or to essential genes for nodule formation in *S. meliloti* (Fogher *et al.,* 1985; Vieille and Elmerich, 1990; Delledonne *et al.,* 1990) were identified. One of these genes complemented the *chvB*$^{-}$ mutant of *A. tumefaciens* for tumour formation. It was speculated that the *chvB* locus participates in root adsorption in *Azospirillum* (Raina *et al.,* 1995). Homologous sequences to the *S. meliloti nodPQ* genes were found to reside on the 90-MDa plasmid in *A. brasilense* (Vieille and Elmerich, 1990), whereas the *nodG*-homologous gene appeared to be chromosomally located (Vieille and Elmerich, 1992). In *S. meliloti*, there is firm evidence that *nodPQ* homologues can function as a sulphate-activating complex for synthesis of sulfur-containing amino acids and sulfation of nod factors (Schwedock *et al*., 1994). Although the function of the *nodPQ* genes in *A. brasilense* still needs to be experimentally

determined, preliminary *in silico* analysis suggests that *nodPQ* might be involved in sulfation of sugar residues in *Azospirillum* (Vanbleu, unpublished results).

3.2 Attachment is a Two-Step Process

Using short-term *in vitro* binding assays, attachment of *Azospirillum* to wheat roots proceeds in two distinct phases, an adsorption phase followed by an anchoring phase (Michiels *et al.,* 1991). This biphasic process is similar to that described for *Agrobacterium* and *Rhizobium* (Matthysse *et al.,* 1981; Smit *et al.,* 1987). In the adsorption step, bacteria adsorb to the roots as single cells in a rapid weak and reversible way. The adsorption step is followed by the anchoring step in which bacterial aggregates are formed that are firmly and irreversibly anchored to the roots. This anchoring phase, characterized by the production of long fibrils, has been observed on roots of several crop plants (Bashan *et al.,* 1991) and is probably the major factor in effective root colonization that ultimately enhances plant growth (Figure 3) (Bashan, 1986; Bashan and Levanony, 1989).

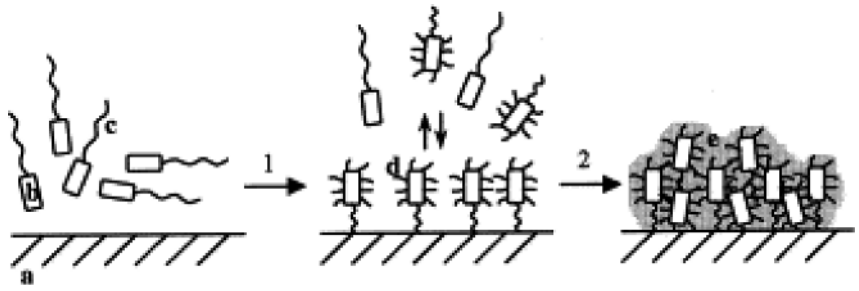

Figure 3. Biphasic attachment process of A. brasilense *to plant root surfaces. Step 1 represents a weak, reversible adsorption mediated by the flagellin of the polar flagellum. Step 2 represents firm, irreversible anchoring, in which extracellular polysaccharides play a role. The letters used refer to the plant root surface (a),* A. brasilense *cells (b), the polar flagellum (c), lateral flagella (d) and extracellular polysaccharides (e). The role of the lateral flagella in the attachment process is not yet clear (reprinted from Steenhoudt and Vanderleyden, 2000, with permission from Elsevier).*

3.3. Role of Flagella

Bacterial flagella serve primarily in locomotion and have rarely been implicated in attachment. However, substantial evidence demonstrates that the polar flagellum of *A. brasilense* plays an important role in the first step of the adhesion process (Croes *et al*., 1993). First of all, a non-adsorbing mutant of *A. brasilense* Sp7 (p90D084) was shown not to form either polar or lateral flagella (Croes *et al.,* 1991). Second, the loss of the flagellar filaments, after heat and acid treatments, eliminated wheat root adsorption by *A. brasilense* Sp7. Third, the adsorption capacity under well-agitated conditions of a completely non-flagellated *A. brasilense* mutant and three

mutants, which lacked the polar flagellar filament protein Fla1, was reduced in comparison to flagellated wild-type bacteria. Finally, purified polar flagella from *A. brasilense* Sp7 were shown to bind to wheat roots. Therefore, the adsorption deficiency of *A. brasilense* mutants lacking the polar flagellum probably reflects the loss of a flagellar-root adhesin. The polar flagellin might function as a non-specific binding adhesion to mediate attachment to various plant species (Vanbleu, 2001).

Some *laf1*::Km mutants of *A. brasilense* Sp7, which are impaired in lateral flagella production, showed no differences from the wild type at the initial stage of root colonization. However, an effect of the mutation on long-term colonization could not be excluded because lateral flagella are believed to enable bacterial movement along the root surface (Moens *et al.,* 1995a).

3.4. Outer Membrane Proteins

Besides the implication of a flagellar protein in the rapid and reversible adsorption step, Burdman *et al.* (2001) demonstrated the role of the major outer-membrane protein (MOMP) of *A. brasilense* Cd as an adhesin in this adsorption step. The gene, *omaA*, encoding the major outer membrane protein of *A. brasilense* Cd, has been identified (Burdman *et al.,* 2000a) and its product was shown to possess high affinity to roots of corn seedlings in an *in vitro* adhesion assay. There seems to be plant specificity in attachment of the MOMP because the MOMP exhibited higher binding affinity towards root extracts of cereals (Burdman *et al.,* 2001) in comparison to legume extracts.

3.5. Polysaccharides

The cell wall of Gram-negative bacteria is quite complex. It consists of a peptidoglycan layer surrounded by the outer membrane. The outer membrane is a phospholipid bilayer, attached to the peptidoglycan by lipoproteins. The outer layer of this membrane consists of lipopolysaccharides (LPS), which are polysaccharides anchored in the outer membrane by a lipid part. Besides LPS, both exopolysaccharides (EPS) and capsular polysaccharides (CPS) are also present on the surface of the bacteria. Capsular polysaccharides (CPS) are referred to as secreted slime polysaccharides that are stuck to the surface of the bacteria. Extracellular polysaccharides (EPS) are also secreted polysaccharides, but they can be found in the supernatant of bacterial cultures. As constituents of the surface of Gram-negative bacteria, these polysaccharides play an essential role in the interaction either between plants and bacterial plant symbionts, *e.g*., *Rhizobium* or between plants and plant pathogens, *e.g*., *Agrobacterium* (reviewed by Vande Broek and Vanderleyden, 1995).

The role of surface polysaccharides in *Azospirillum*-plant root colonization was initially examined by isolating Tn5-induced mutants of *A. brasilense* that had lost their fluorescence on agar medium containing Calcofluor (Cal$^-$ mutants) (Michiels *et al.,* 1990). Calcofluor is a fluorescent dye that is specific for β-linked polysaccharides. *A. brasilense* Cal$^-$ mutants were shown to produce comparable

amounts of EPS to the wild-type strain, whereas Leigh *et al.* (1985) reported a diminished EPS production in *S. meliloti* Cal$^-$ mutants. Thus, the nature of the Calcofluor-binding polysaccharide(s) in *A. brasilense* remains unknown. The Cal$^-$ mutants were affected in flocculation (Michiels *et al.,* 1990) and exhibited a reduced anchoring capacity to wheat roots (Michiels *et al.,* 1991) but retained the wild-type adsorption capacity. The Tn5 insertions were localized on the *A. brasilense* chromosome.

Other studies revealed major differences in surface composition between *A. brasilense* Sp7 and Sp245 (De Troch *et al.,* 1992). The polysaccharides of Sp7 and Sp245 are different (Konnova *et al.,* 1990; Konnova *et al.,* 1992). Monoclonal antibodies that react with the polar flagellum of Sp7 do not react with Sp245. Also, monoclonal antibodies that react with LPS of Sp7 do not react with Sp245. These differences might be related to a difference in interaction with the plant.

In a second approach, loci of *A. brasilense*, which appear to be involved in EPS synthesis, were identified through complementation of *S. meliloti exoB* and *exoC* mutants (Michiels *et al.,* 1988). Two genes in *A. brasilense*, *exoB1* and *exoB2* were isolated and both seemed to play a role in galactose metabolism. They encode an UDP-glucose 4'-epimerase, an enzyme that converts UDP-glucose into UDP-galactose. UDP-galactose is incorporated into EPS, CPS, and LPS (De Troch *et al.,* 1994). The presence of two genes coding for UDP-glucose 4'-epimerase is unusual in bacteria and it suggests that *A. brasilense* has a different system for regulating the UDP-glucose and UDP-galactose levels. The presence of multiple genes that regulate the UDP-galactose level may allow the bacterium to respond quickly to environmental changes by altering its polysaccharide synthesis. It may also be linked to cyst formation. However, these hypotheses need to be confirmed. The *A. brasilense exoC* locus encodes a phosphomannomutase essential for the conversion of GDP-mannose-6-phosphate to GDP-mannose-1-phosphate. The *A. brasilense exoB1* and *exoC* loci were mapped to the 90-MDa plasmid (Michiels *et al.,* 1989; Croes *et al.,* 1991), whereas the *exoB2* locus is chromosomally located (De Troch *et al.,* 1995).

No differences were found between *A. brasilense exoBC* mutants and the wild-type strain in either Calcofluor binding or EPS amount, indicating that the EPS synthesized by the *A. brasilense exo* loci is not the Calcofluor-binding polysaccharide (Michiels *et al.,* 1988). Also, no difference in primary colonization of wheat roots (Vande Broek *et al.,* 1998) has been observed between the *A. brasilense* wild-type strain and the *exoB1* and *exoC* mutants.

A regulatory gene, *flcA*, which controls flocculation and production of capsular polysaccharides, has been identified in *A. brasilense* Sp7. *flc*A mutants were impaired in root-surface colonization (Pereg-Gerk *et al.,* 1998; see section 4.3).

3.6. Pili

Pili are non-flagellar filamentous surface appendages that are composed of protein subunits. A wide variety of Gram-negative bacteria are able to produce pili. Type IV pili play an essential role in mediating bacterial adherence to cell surfaces and

are virulence factors in *N. gonorrhoeae*, *N. meningitidis*, *P. aeruginosa*, and *Moraxella bovis* (Strom and Lory, 1993; Hahn, 1997). In addition, type IV pili play a role in cell-cell interaction, twitching motility, and biofilm formation (Strom and Lory, 1993; Wall and Kaiser, 1999). Some type IV pili also serve as receptors for bacteriophages. In the diazotrophic endophyte *Azoarcus* sp. strain BH72 (Reinhold *et al.,* 1986), adhesion to plant roots was mediated by type IV pili. Two genes, *pilAB*, that are involved in pilus formation have been isolated (Dörr *et al.,* 1998).

K. pneumoniae expresses two types of fimbriae, type 1 and type 3, which have been assigned adhesion properties (Korhonen *et al.,* 1983; Haahtela *et al.,* 1985). Type 1 pili are also involved in infections and in adhesion to animal and human tissues.

3.7. Plant receptors

Several authors have suggested that root lectins are involved in the process of root colonization of azospirilla (Umali-Garcia *et al.,* 1980; Patriquin *et al.,* 1983; Elmerich, 1984). Lectins are proteins that recognize and reversibly bind to specific sugar chains of glycosylated molecules (Goldstein *et al.,* 1980). The best-studied grass lectin is wheat germ agglutinin (WGA) (LeVin *et al.,* 1972), which is present on the entire surface of root seedlings and on the root tips of adult wheat plants (Mishkind *et al.,* 1983). It may, therefore, be a specific site for putative receptors and may contribute to bacterial adhesion to the root surface, leading to wheat-root colonization.

Del Gallo *et al.* (1989) reported the ability of different *A. brasilense* and *A. lipoferum* strains to bind to WGA, indicating the occurrence of specific sugar-bearing receptors on the cell surface. However, the molecular genetics of these surface structures remain to be developed. WGA has been proposed to act as a signal molecule in the association between *Azospirillum* and wheat roots. Binding of WGA to *A. brasilense* Sp245 enhances N_2 fixation, ammonium excretion, and indole-3-acetic acid (IAA) biosynthesis (Antonyuk *et al.,* 1993; Antonyuk and Ignatov, 2001), and promotes protein synthesis. A similar enhancement in nitrogen-fixation capability in the presence of WGA has been shown for *A. lipoferum* (Karpati *et al.,* 1999). A putative WGA-binding receptor, a 32-kDa protein, was detected in the cell capsule.

Castellanos *et al.* (1998) extracted cell-wall proteins from *A. brasilense* and *A. lipoferum* strains and showed their lectin-like activities but it is yet to be demonstrated whether these lectins are involved in the recognition and colonization of the root surface.

4. RHIZOSPHERE COMPETENCE

Rhizosphere competence is another important requirement for efficient root colonization. Bacteria must be able to survive in the soil and adapt to environmental changes and stress conditions in the rhizosphere. In addition to their ability to move towards the most favourable niche and to attach to the plant-root

system, associative bacteria are very versatile organisms. Azospirilla, for example, can use a variety of carbon sources for energy and biomass production. Azospirilla are able to use molecular nitrogen, nitrate, and ammonium ions for growth and also to perform anaerobic respiration with nitrate (Bothe *et al.,* 1981; Hartmann and Zimmer, 1994). The bacteria produce both bacteriocins to inhibit growth of other bacteria and siderophores for efficient Fe acquisition (Michiels *et al.,* 1989). Under stress conditions, azospirilla can differentiate into cysts, which make them more resistant (Sadasivan and Neyra, 1985) and they are able to synthesize and store poly-β-hydroxybutyrate (PHB) as a carbon reserve polymer inside the cells (Tal and Okon, 1985; Okon and Itzigsohn, 1992).

We will highlight the different factors that are important in rhizosphere competence, however, neither nitrogen fixation nor ammonium assimilation will be covered in this chapter as both are discussed into detail in Chapter 3 of this volume. Furthermore, from the wide variety of carbon-utilization pathways available in diazotrophs, only the metabolism of complex carbohydrates and biopolymers will be reviewed here.

4.1. Nitrogen Metabolism

4.1.1. Nitrate Assimilation

Nitrate is a major source of inorganic nitrogen for many bacteria. Nitrate assimilation involves transport of nitrate into the cell and the reduction of nitrate to ammonium by the sequential action of nitrate reductase and nitrite reductase ($NO_3^- \rightarrow NO_2^- \rightarrow NH_4^+$). Genes involved in nitrate assimilation have been characterized in the associative diazotrophs, *Klebsiella oxytoca*, *Azotobacter vinelandii* (Lin and Stewart, 1998), and *Azospirillum brasilense* Sp245 (Steenhoudt, 2000; see also *Nitrogen Fixation in Agriculture, Forestry, Ecology, and the Environment*, volume 4 of this series).

In most bacterial species studied so far, nitrate is transported into the cells by an active transport system that belongs to the group of ABC-type transporters (ATP-binding cassette) (Higgins, 1992). In *Klebsiella oxytoca* and *A. brasilense* Sp245, genes encoding the components of the ABC transporter have been identified (Figure 4). The *nasFED* genes code for a periplasmic binding protein-dependent system that is energized by ATP hydrolysis. Growth tests revealed that the *nasFED* genes are essential for nitrate transport and participate in nitrite transport as well. However, observations with deletion mutants suggested the existence of a NO_2^- specific-transport system (Lin and Stewart, 1998; Steenhoudt, 2000).

Once NO_3^- has entered the bacterial cell, it is reduced in two consecutive steps to ammonium. The assimilatory nitrate reductase catalyses the 2-electron reduction of NO_3^- into NO_2^-. Purified assimilatory nitrate reductase of *A. vinelandii* is a single soluble 105-kDa polypeptide associated with a molybdenum cofactor and a [4Fe-4S] cluster. Flavodoxin appears to be the *in vivo* electron donor. In *K. oxytoca*, two polypeptides are needed to constitute the assimilatory nitrate reductase. The *nasA* gene encodes the catalytic subunit of this enzyme, which binds the molybdenum cofactor, whereas NasC functions as an electron-transfer subunit

between a NAD(P)H-reducing unit and the catalytic component, NasA, of the assimilatory nitrate reductase. In *A. brasilense* Sp245, only one ORF was identified as being necessary for the reduction of NO_3^- to NO_2^- (Steenhoudt, 2000). NasA displays features typical of molybdoenzymes (a molybdenum cofactor-binding domain and a FeS-binding motif; Wootton *et al.,* 1991) but lacks recognizable FAD- and NAD(P)H-binding domains. It shares a C-terminal extension with the NasA of *K. oxytoca*, which is similar to [2Fe-2S] cluster-containing proteins involved in electron transfer. Therefore, it is inferred that this extension serves an electron-transfer role (Lin and Stewart, 1998).

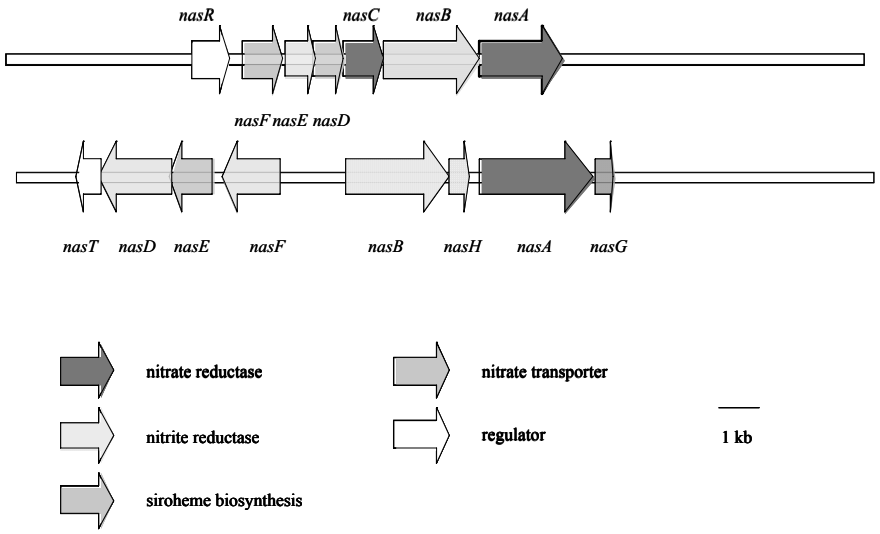

Figure 4. Genetic organization of the NO_3^- assimilation gene clusters of K. oxytoca (top) and A. brasilense Sp245 (bottom). Differential shading indicates the function of the gene products (courtesy of Steenhoudt, 2000).

The 6-electron reduction of NO_2^- to NH_4^+ is catalysed by the assimilatory nitrite reductase. The NAD(P)H-dependent assimilatory nitrite reductase, NasB, in *K. oxytoca* consists of a single polypeptide. In *A. brasilense* Sp245, it is assumed that the NasBH proteins constitute two subunits of an assimilatory NAD(P)H-dependent nitrite reductase.

On the whole, nitrate assimilation genes are organized in clusters. Moreover, evidence indicates that these clusters comprise operons in *K. oxytoca* (Lin *et al.,* 1994), *A. vinelandii* (Ramos *et al.,* 1993), and *A. brasilense* Sp245 (Steenhoudt, 2000). Transcription of the genes was shown to be subject to dual control. General control (Ntr) involves repression of transcription by the preferred fixed-N source,

ammonium, whereas pathway-specific control induces transcription in response to the availability of NO_3^- and/or NO_2^- (Lin and Stewart, 1998; Steenhoudt, 2000).

4.1.2. Denitrification and Nitrate Dissimilation

Under O_2-limiting conditions, bacteria can utilize nitrate as an alternative respiratory electron acceptor. This anaerobic respiration uses either membrane-bound or periplasmic enzyme complexes to reduce nitrate.

A thorough genetic analysis of the enzymes involved in anaerobic respiration has been performed only for *A. brasilense* Sp245. A periplasmic dissimilatory nitrate reductase-gene cluster, *napABC*, was isolated (Steenhoudt *et al*., 2001) and suggested to be a molybdoenzyme. No membrane-bound respiratory nitrate reductase seems to be present in *A. brasilense* Sp245. Furthermore, the primary function of the *nap*-gene cluster in *Azospirillum* is not coupled with the generation of a proton motive force because wild-type cells do not grow on NO_3^- when O_2 is absent. The nitrate-reductase enzyme might be involved in redox balancing in the bacterial cell and might serve a role in dissipating excess reducing equivalents, as is the case for *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, and *Ralstonia eutropha* (Richardson, 2000).

Other reports, however, indicated that *A. lipoferum* and *A. brasilense* strains could grow on nitrate and other fixed-N sources, such as NO_2^- and N_2O, as the terminal electron acceptor. Reduction is then coupled to the generation of a proton motive force that supports growth of *A. brasilense* under anaerobic conditions (Penteado Stephan *et al.,* 1984; Zimmer *et al.,* 1984). In *A. brasilense* Sp7, growth yield with nitrate is two-thirds lower than that with O_2, indicating that O_2 is the preferred electron acceptor. Determination of the growth yield also indicated that *Azospirillum* grows as efficiently with N_2O as with O_2, whereas the growth yield with nitrite was one third lower (Danneberg *et al.,* 1989).

Both the respiratory nitrite reductase and nitrous oxide reductase of *A. brasilense* Sp7 have been characterized respectively as a cytochrome cd_1-nitrite reductase (*nirS*) and a multi-Cu enzyme (*nosZ*) (Danneberg *et al.,* 1986; Danneberg *et al.,* 1989; Lalande and Knowles, 1987; Zimmer *et al.,* 1995). In other *A. brasilense* and *A. lipoferum* strains and in *A. halopraeferens*, the *nosZ* and *nirS* genes have also been localized by PCR amplification (Rösch *et al.,* 2002).

4.2. Metabolism of Biopolymers and Complex Carbohydrates

4.2.1. Pectin

Pectins are a complex group of heteropolysaccharides, consisting of a backbone of galacturonic acid, with either esterified or non-esterified carboxylgroups. Given the complex structure of pectic substances, several types of pectinolytic enzymes are required for their degradation. Pectins are components of the cell wall, together with cellulose, hemicellulose and proteins. Pectinolytic activity has been reported in *K. pneumoniae* (Chaterjee *et al.,* 1979) and in *A. irakense* strains (Khammas and

Kaiser, 1991). Only *A. irakense* is able to grow with pectin as sole carbon source (Khammas and Kaiser, 1991). The presence of pectinolytic enzymes in endophytic diazotrophic bacteria may suggest that an active penetration process is involved in the infection mechanism of these bacteria. Kovtunovych *et al.* (1999) correlated an increase in internal colonization with increased levels in pectate lyase, which suggests that colonization occurs *via* lysed pectin layers.

Two types of pectinolytic enzymes are present in *A. irakense*; an inducible Ca^{2+}-dependent pectate lyase and a pectin methyl esterase (Khammas and Kaiser, 1991). Bekri (1998) isolated genes encoding pectate lyase and polygalacturonase activity. *A. irakense* pectate lyase (*pelA*) showed no homology with other known bacterial, plant, or fungal pectinases (Bekri *et al.,* 1999). Therefore, the *A. irakense* PelA protein defines a new family of pectate lyases. In addition, *A. irakense* seems to contain additional pectinolytic gene(s) because a mutant with an inactivated *pelA*-gene was still able to grow in medium containing pectin as sole carbon source (Bekri *et al.,* 1999). The synthesis of pectin lyase is thought to be well regulated; its synthesis is enhanced in the presence of pectin and is growth-phase dependent. Heterologous expression of the *A. irakense pelA* gene is higher as compared to homologous expression, which suggests strict regulation of pectinolytic activity in *A. irakense*, probably through some negative regulatory gene products.

4.2.2. Cellulose

Cellulose is composed of D-glucopyranosyl units linked by β-(1,4) bonds. For its microbial conversion, at least three classes of hydrolytic enzymes are thought to participate (Reinhold-Hurek *et al.,* 1993). Endoglucanase and exoglucanase activity has been demonstrated in *Azoarcus* sp. strain BH72 (Reinhold-Hurek *et al.,* 1993). However, although *Azoarcus* possesses a cellulolytic system, it cannot grow on cellulose or on its breakdown products. This might suggest that cellulases are involved in the infection process of grass roots by azoarci. The enzymes, β-glucosidase and cellobiohydrolase, were observed in *A. irakense* (Bekri, 1998).

4.2.3. Aryl-β-glucosides

A. irakense KBC1 naturally assimilates β-glucosides, such as cellobiose, salicin and arbutin. Two β-glucosidases, SalA and SalB, are implicated in the assimilation of the aryl-β-glucosides (Faure *et al.,* 1999). Either SalA or SalB is sufficient for growth on salicin as sole carbon source, indicating a functional redundancy of the genes for the utilization of salicin (Faure *et al.,* 1999). Upstream of the *salAB* genes, two additional ORFs have been found (Somers *et al.,* 2000). The *salC* gene encodes a putative outer-membrane transporter and forms an operon with *salAB*. The *salR* gene, which is oriented divergently, encodes a transcriptional repressor of the operon. Expression of the *sal* operon requires the presence of aryl-β-glucosides and its expression is enhanced by the addition of a simple carbon source. Moreover, the *sal* operon is sensitive to the hydrolysis products of salicin and arbutin. This may indicate that additional regulatory systems, in addition to SalR, control the *sal* operon (Somers *et al.,* 2000).

4.2.4. Poly-β-hydroxybutyrate (PHB)

Poly-β-hydroxybutyrate (PHB) is described as a reserve polymer commonly found in prokaryotes. However, in most cases, the polymers are actually polyhydroxyalkanoates (PHA), which are copolymers containing different alkyl groups at the β-position (Madison and Huisman, 1999). The biosynthesis and degradation of PHB in *Azotobacter beijerinckii* and *A. brasilense* Cd occur *via* a cyclic mechanism (Stevenson and Socolofsky, 1966; Senior and Dawes, 1973; Dawes, 1986; Tal *et al.,* 1990), which is similar in both organisms. Three enzymes are involved in PHB synthesis; a β-ketothiolase, an acetoacetyl-coenzyme A reductase, and a PHB synthetase (Manchak and Page, 1994).

In *A. brasilense* Sp7, the genes encoding these enzymes, *phbABC*, have been isolated (Kadouri *et al.,* 2002) and located on the chromosome. A *phbC* mutant, which was impaired in PHB production (Figure 5), was shown to have a decreased survival rate under starvation conditions, while exhibiting an increase in motility, production of polysaccharides, and adhesion to roots (Kadouri *et al.,* 2002). These findings agree with earlier physiological observations by Tal and Okon (1985). They reported control of the PHB synthesis in *Azospirillum* by both O_2 pressure and C/N ratio in the medium (Tal and Okon, 1985). In terms of genetics, Sun *et al.* (2000; 2002) showed that the regulation of PHB biosynthesis in *A. brasilense* Sp7 involves the nitrogen-regulatory genes, *glnB*, *glnZ*, *glnD*, *ntrB*, and *ntrC*.

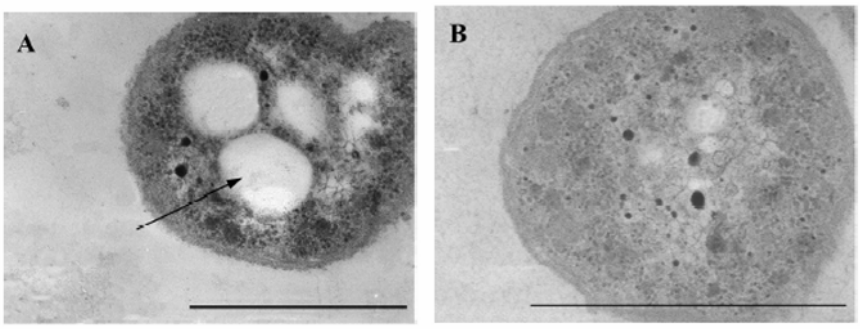

Figure 5. Electron micrographs of thin sections of A. brasilense *(A) and of the phbC mutant (B). The arrow indicates PHB granules. Bars 1 μm. (Repinted from Kadouri* et al*., 2002, with permission of American Society for Microbiology).*

The gene cluster *phbBAC*, encoding the enzymes involved in PHB biosynthesis, have been cloned and identified in *Azotobacter* sp. strain FA8 (Pettinari *et al.,* 2001). Three genes in *A. vinelandii*, coding for β-ketothiolase were identified (Segura *et al.,* 2000), of which only the *phbA* gene was shown to be involved in PHB accumulation. The *phbR* gene, which is located upstream and in the opposite

direction to *phbBAC*, encodes PhbR, a transcriptional activator that is a member of the AraC family of activators (Peralta-Gil *et al*., 2002). The *cydR* gene has been demonstrated to control PHB synthesis in *A. vinelandii* (Wu *et al.*, 2001) because overexpression of both β-ketothiolase and acetoacetyl-CoA reductase occurs in a *cydR* mutant. In addition to *cydR*, it was shown that a mutation in *gacS*, coding for a sensor kinase of a two-component regulatory system, plays a role in regulating PHB synthesis and in alginate production (Castañeda *et al.,* 2000). This might indicate that *gacS*, together with *gacA*, its cognate-response regulator, is part of a global system regulating polymer synthesis. Further, a mutation in *ptsP* (homologous to *E. coli ptsP*), which encodes a homologue of enzyme I of the phosphoenolpyruvate-sugar phosphotransferase system, leads to a failure in PHB accumulation (Segura and Espín, 1998).

In *A. brasilense* Sp7, the gene *phaZ*, which is involved in depolymerization of PHB, has been isolated and sequenced (Kadouri *et al*., 2003). A *phaZ* mutant was unable to degrade PHB and, therefore, unable to use the PHB polymer as an energy and carbon source under starvation conditions. Also, a *phaZ* mutant was reduced in its ability to tolerate stress conditions, indicating the importance of a functional PHB cycle in stress endurance.

4.3. Cyst Formation

Under environmental stress, *Azospirillum* and *Azotobacter* species are capable of cyst and floc (macro-aggregate) formation, both of which significantly improve survival. Stress conditions can be aging, toxic metals, ultra-violet or γ-irradiation (Lamm and Neyra, 1981), or water stress (Bashan *et al.,* 1991). Flocculation also occurs in liquid cultures of *Azospirillum* when either fructose or β-hydroxybutyrate is the carbon source (Sadasivan and Neyra, 1985; Bleakley *et al.,* 1988).

Vegetative cells differentiate into non-motile, encapsulated C-forms (cysts) that contain abundant poly-β-hydroxybutyrate granules (Stevenson and Socolofsky, 1966; Eskew *et al*., 1977; Tarrand *et al*., 1978; Sadasivan and Neyra, 1987; Pereg-Gerk *et al*., 1998). As a result, cells aggregate firmly within a polysaccharide matrix, forming large macroscopic clumps (Sadasivan and Neyra, 1985; Bastarrachea *et al*., 1988). They are not dormant because they are capable of fixing nitrogen and have high nitrate reductase and glutamate synthase activity (Bashan and Holguin, 1997). Several suggestions have been published concerning the involvement of exopolysaccharides and proteins in the aggregation of *Azospirillum*. Non-fluorescent mutants that are impaired in EPS synthesis, flocculation, and cyst formation have been obtained, indicating that Calcofluor-binding polysaccharides are needed for cell aggregation (Michiels *et al*., 1990). Sadasivan and Neyra (1985) related floc formation in *A. brasilense* Sp7 and *A. lipoferum* to β-linked EPSs.

Genetic complementation of a spontaneous mutant of *A. brasilense* Sp7, designated Sp7-S, impaired in flocculation and colonization of the root surface (Katupitiya *et al*., 1995), led to the identification of a regulatory gene, *flcA*. The deduced protein product shares similarity with the C-terminal domain of a large number of transcriptional regulators of the LuxR-UhpA family (Pereg-Gerk *et al*.,

1998). Most of these are response regulators, suggesting that FlcA could be part of a similar two-component regulatory system. No gene encoding for a sensor component is found in the vicinity of *flcA*. Construction of *A. brasilense* Sp7 derivatives carrying Tn5 insertions in the *flcA*-coding sequence resulted in mutant strains that remain in the vegetative form during the flocculation process. This showed that the same gene was responsible for the control of flocculation and cyst formation. The *flcA* gene was required for the aggregation of the cells, formation of a thick outer coat and capsular material, and the abundant secretion of polysaccharides that form a net surrounding the bacteria, but it was not required for the accumulation of PHB granules and the brown pigment found in cysts. In association with wheat roots, the *flcA*-Tn*5* mutants presented a morphology resembling their vegetative form and this was correlated with a strong decrease of root-surface colonization as compared to that observed with the wild type (Pereg-Gerk *et al*., 1998).

Burdman *et al*. (1998) showed a strong correlation between the EPS concentration produced by different strains of *A. brasilense* and their extent of aggregation. Arabinose, present in extracellular polysaccharides, may play an important role in determining the aggregation capability of *A. brasilense* (Castellanos *et al*., 1998; Burdman *et al*., 2000b). Indeed, no arabinose was detected in polysaccharides from a *flcA*-mutant strain (Burdman *et al*., 2000b). The outer-membrane proteins (OMPs) are involved in the aggregation process of cells (Burdman *et al*., 1999). It is hypothesized (Burdman *et al*., 2000b) that the OMPs involved in aggregation are constitutively expressed and that the differences in the extent of aggregation is related to the composition of the extracellular polysaccharides.

Azotobacter vinelandii produces alginate, an exopolysaccharide, which is a component in the cyst capsule (Sadoff, 1975). The genes involved in alginate biosynthesis (Gacesa, 1998) are clustered and organized in three operons. *algU*, a putative σ factor homologous to σ^E has been shown to control alginate production and to be involved in the encystment process independent of its role in alginate synthesis (Moreno *et al.,* 1998). Inactivation of the response regulator, *algR*, completely impairs the capacity to form mature cysts (Núñez *et al.,* 1999). A mutation in *gacS*, a gene homologous to a sensor kinase of the two-component regulatory systems in *Pseudomonas*, impairs encystment (Castañeda *et al.,* 2000).

4.4. Plant-Inducible Genes

The influence of azospirilla on the host plant has been extensively studied. The plant stimulatory effect exerted by *Azospirillum* is attributed to both N_2 fixation and auxin production (see Chapters 6 and 7 of this volume).

The influence of the plant on bacterial gene expression in *A. brasilense* has also been demonstrated (Van Bastelaere *et al*., 1999). An acidic 40-kDa protein, SbpA, was strongly induced in *A. brasilense* by addition of root exudates of several plant species to the growth medium. The induction in *A. brasilense* was shown to be independent of the plant species tested whereas, in *A. lipoferum*, induction was only

observed with maize-root exudates. The SbpA protein showed high similarity to the ChvE protein of *A. tumefaciens* and seems to be involved in the uptake of D-galactose by a protein-binding high-affinity uptake system. This protein plays a role in the chemotactic response of *A. brasilense* towards several sugars (Croes *et al.,* 1993).

Apart from the involvement of SbpA in a galactose-transport system, not much is known about nutrient-uptake systems in *Azospirillum*. A phosphotransferase system (PTS), using phosphoenolpyruvate as energy source (Dutta Gupta and Ghosh, 1984), was shown to be involved in the uptake of sugars. Uptake of siderophores by a specific outer-membrane protein was demonstrated in *A. brasilense* RG (Bachhawat and Ghosh, 1987a; Bachhawat and Ghosh, 1987b). In *A. brasilense* Sp7, an ATP-binding cassette (ABC) transporter, GlnHPQ, is suggested to mediate the uptake of either an unidentified polar amino acid or opine (Van Bastelaere, 1996).

4.5. Antagonism

To survive in the rhizosphere, bacteria must prevent competitors from growing. Bacteria may compete in different ways through either the production of antibiotics or competition for an essential nutrient, like iron (see Chapter 8 of this volume). Even though some evidence is available on antagonistic mechanisms, the genes encoding these antagonistic mechanisms have not yet been identified.

4.5.1. Bacteriocins

Many azospirilla have the capacity to produce bacteriocins (Oliveira and Drozdowicz, 1981; 1987; Skorupska *et al.,* 1985). The bacteriocins described here are proteinaceous inhibitors of bacteria or fungi. Yet, these bacteriocins have not been identified. Bacteriocins, which inhibited the growth of several indicator bacteria, have been isolated from azospirilla species (Tapia-Hernández *et al.*, 1990). Bacteria belonging to the genus *Azotobacter* have been found to be antagonistic against *Botrytis cinerea*. These bacteria synthesize an antifungal compound of low molecular weight that overall inhibits the production of conidia by the fungus (Doneche and Marcantoni, 1992)

4.5.2. Siderophores

Siderophores are low molecular-weight molecules that are produced under Fe-limiting conditions and which efficiently bind iron. Soil microorganisms secrete siderophores, which bind iron and transport it back to a microbial cell (Neilands, 1981). The iron-siderophore complex is recognised by specific membrane receptors (Neilands, 1982). This Fe-binding system has been reported for *A. lipoferum* (Saxena *et al.,* 1986), *A. brasilense* (Bachhawat and Ghosh, 1987a), and *A. vinelandii* (Knosp *et al.,* 1984; Demange *et al.,* 1988; Page and von Tigerstrom, 1988).

A. lipoferum M produces catechol-type siderophores under iron-starved conditions (Shah *et al.,* 1992). Similarly, *A. brasilense* produces a catechol-type

siderophore, called spirilobactin (Bachhawat and Ghosh, 1987a). Spirilobactin-dependent iron uptake is an active process, requiring a proton gradient as the driving force. *A. lipoferum* is also able to take up iron *via* this spirilobactin-siderophore, suggesting the existence of a spirilobactin-type high-affinity iron-transport system in *A. lipoferum*.

A. vinelandii produces three different siderophores: a yellow-green fluorescent compound azotobactin (Knosp *et al.,* 1984); and the catechols azotochelin (Knosp *et al.,* 1984) and aminochelin (Page and von Tigerstrom, 1988). *A. vinelandii* also expresses four different outer-membrane proteins in response to iron limitation (Page and Huyer, 1984) and these probably serve as siderophore receptors. However, no genes or mutants have been identified.

The specificity and high affinity for iron of these transport systems may enable diazotrophs to compete with other organisms for the available iron, thereby displacing other microorganisms, lacking such an efficient siderophore system. This was shown for *A. brasilense* (Tapia-Hernández *et al.,* 1990) and *A. lipoferum* M (Shah *et al.,* 1992) where the produced siderophores exhibited antimicrobial activity against various bacterial and fungal isolates. However, *A. vinelandii* siderophores appeared to promote growth of the phytopathogenic *A. tumefaciens* and *Erwinia carotovora* (Page and Dale, 1986). Therefore, extensive further research is needed to elucidate this antagonism.

4.6. Other Mechanisms

Pseudomonas putida GR12-2, reported as a nitrogen-fixing rhizospheric bacterium isolated from *Brassica campestris* (Lifshitz *et al*., 1986), is able to deal with cold temperatures. The basis of its freezing resistance is the synthesis and secretion of an antifreeze protein into the growth medium (Sun *et al.*, 1995; Xu *et al.,* 1998). The purified antifreeze protein displays low-level ice-nucleation activity. It regulates the formation of ice crystals outside the bacterium, thereby protecting it from damage and permitting the bacterium to thrive under these adverse conditions.

Osmotolerance occurs with *Azospirillum*. Both *A. brasilense* and *A. halopraeferens* can grow and fix nitrogen under osmotic stress by using osmoprotectants available in the rhizosphere (Riou and Le Rudulier, 1990). Some *A. brasilense* strains (Rai, 1991) show salt tolerance as does the halotolerant *A. halopraeferens* (Reinhold *et al.,* 1987). Hartmann *et al.* (1991) concluded that, in the genus *Azospirillum*, tolerance towards high concentrations of NaCl, sucrose, or polyethylene glycol increases in the order *A. amazonense*, *A. lipoferum*, *A. brasilense*, and *A. halopraeferens*. The osmotolerance of *A. irakense* lies between that of *A. brasilense* and *A. halopraeferens* (Khammas *et al.*, 1989).

5. CONCLUSIONS

Establishing a beneficial bacteria-plant root interaction requires an efficient association of plant growth-promoting rhizobacteria with the host roots. This

association enables the bacteria to get into close contact with the host root system and to compete with other rhizobacteria. The best-characterized associative diazotrophic bacteria so far are azospirilla. However, as a successful *Azospirillum*-plant association does not result in an easily detectable plant phenotype, direct screening of *Azospirillum* mutants that are defective in the interaction process is difficult. Therefore, genetic studies of the *Azospirillum*-plant interaction rely on indirect approaches. Genes possibly involved in plant interaction are traced either by characterization of mutants defective in a phenotype that is thought to be involved in plant association or by isolation of putative plant-interaction genes with conserved structural or functional homology with already identified plant-interaction genes of other plant associative bacteria. Recently, an '*in vitro* expression technology' strategy (IVET) was used to identify the mechanisms which enable *P. stutzeri* A15 to colonize and infect rice roots (Rediers *et al*., 2003). IVET, originally devised for pathogenic bacteria, is a 'promoter-trapping' technique that traces bacterial promoters specifically active during interaction with the host.

Different visualization methods have been developed for *in situ* localization of *A. brasilense* in the rhizosphere. Two methods, which are used for *Azospirillum*-wheat root-colonization determination, rely on the *in situ* staining of bacteria, constitutively expressing either the *E. coli gusA* gene (Vande Broek *et al.,* 1993) or *lacZ* gene (Arsène *et al*., 1994). *Azospirillum*-wheat root colonization was also determined by hybridization with fluorescently-labeled rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy (Assmus *et al*., 1995). In addition, the green fluorescent protein (GFP) and GFP colour variants were used as a bioreporter in monitoring the colonization of wheat roots by *A. brasilense* strain FP2 (Ramos *et al*., 2002) and in visualizing the rice root colonization by *A. irakense* KBC1 and *A. brasilense* Sp7, respectively (Zhu *et al*., 2002). Monitoring the expression of putative *Azospirillum*-plant interaction genes during plant association by the use of reporter genes, and the application of strains carrying mutations in those genes, greatly facilitates the molecular analysis of the plant association and may lead to a better understanding of the bacterial determinants controlling this association.

The genus *Azospirillum* comprises predominantly root surface-colonizing bacteria, with only a few strains that can enter the first layers of root system. However, the plant-root interaction of other associative nitrogen-fixing bacteria, like *G. diazotrophicus*, *H. seropedicae* and *Azoarcus*, clearly displays an endophytic character (Döbereiner *et al*., 1995). The efficient plant-root association and the invasive potential of a plant growth-promoting rhizobacterium might be of great importance for establishing a beneficial bacterium-plant interaction and studies that deal with the way these endophytic diazotrophs internally colonize plant roots could significantly contribute to the development of improved plant growth-promoting rhizobacteria, including *Azospirillum*.

Of course, complete sequencing of the genome of several diazotrophs will provide a full insight in the genetic organization of these organisms. The 90-MDa plasmid of *A. brasilense* Sp7 has been sequenced and annotated (Vanbleu, unpublished data). *In silico* analysis indicates the importance of this plasmid in cell-envelope biogenesis and outer-membrane constitution and so its essential

function in the survival of the bacterium. Furthermore, a draft version of the *Azotobacter vinelandii* genome is already available and sequencing of several other diazotrophic genomes is in process. It is clear that the availability of these complete genome sequences will increase our knowledge on bacterial working mechanisms tremendously and will provide us with the means to unravel important pathways, some of which are yet unknown.

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Chapter 6

MICROBIAL PRODUCTION OF PLANT HORMONES.

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1. DISCOVERY OF PHYTOHORMONES

Plant hormones are signal molecules that act as chemical messengers to control plant growth and development. Aside from their role in plant response to changes in environmental conditions, hormones are also the principal agents that regulate expression of the intrinsic genetic potential of plants. A phytohormone is an organic substance synthesized in defined organs of the plant that can be translocated to other sites, where it triggers specific biochemical, physiological, and morphological responses. However, phytohormones are also active in the tissues where they are produced. In addition, numerous soil bacteria and fungi also produce phytohormones. The commonly recognized classes of phytohormones, regarded as the "classical five", are: the auxins; gibberellins; cytokinins; abscisic acid; and ethylene.

The discovery of auxins during the nineteenth century was the outcome of experiments on phototropism and geotropism (see Moore, 1979). In 1880, Charles Darwin reported on the phenomenon by which the plants bent toward the sunlight in a book entitled "*The Power of Movement of Plants*". Several years later, by 1926, the Dutch botanist Frits W. Went discovered auxin and described a bioassay for its quantitative detection by "the *Avena* coleoptile curvature test". Although Went had succeeded in isolating auxin, he was not able to purify the active compound to establish its chemical structure. In 1934, the biochemists, Kögl, Haagen-Smit and Erxleben, obtained an active substance from urine, indole-3-acetic acid (IAA), which was found to be identical to auxin (Figure 1). Finally, K.V. Thimann isolated IAA from cultures of the fungus *Rhizopus suinus* in 1935. The first generally accepted report of the occurrence of IAA in a higher plant was published by

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Haagen-Smit *et al*. in 1946 (see Moore, 1979). Since then, there have been an increasing number of reports of the occurrence in plants of IAA and related compounds, such as indole-3-butyric acid (IBA; Figure 1), 4-chloro-IAA, and conjugated IAA forms, as well as non-indolic compounds, such as phenylacetic acid, which displays weak auxin activity (see Normanly *et al.,* 1995).

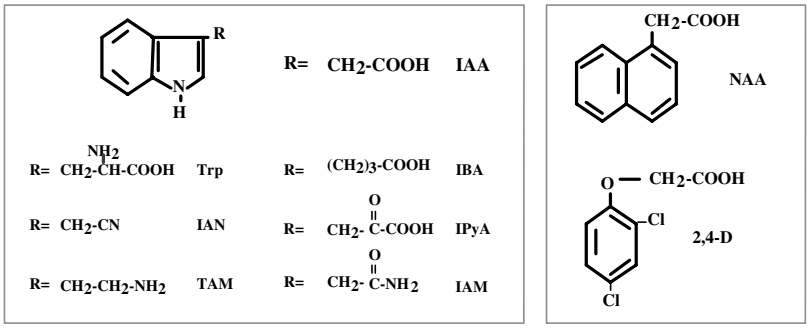

Figure 1. Chemical structure of indole-3-acetic acid (IAA) and precursor compounds and of synthetic auxins NAA and 2,4-D. Trp, tryptophan; IBA, indole-3-butyric acid; IPyA, indole-3-pyruvic acid; IAM, indole-3-acetamide; IAN, indole-3-acetonitrile; TAM, tryptamine; NAA, naphtylacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Research on the gibberellins (GAs) stems from the work of E. Kurosawa (see Moore, 1979). He is credited for having discovered GA in 1926, producing the "bakanae" effect (pathological longitudinal growth) in rice and maize seedlings, when treated with spent-culture medium from the fungus *Gibberella fujikuroi*. Yabuta and Sumiki, in 1938, isolated and crystallized two biologically active substances, which they named “gibberellins A and B”. Thereafter, by 1956, GAs were shown to be natural components of plants tissues both by West and Phinney in the USA and by Radley in England (see Moore, 1979). It then became apparent that these compounds were not merely an interesting group of fungal metabolites but also endogenous regulators of growth and development of plants. Up to now, about 125 different GAs have been characterized (see Crozier *et al.,* 2001). The GAs are divided into two groups: the C_{20}-GAs; and the C_{19}-GAs The C_{20}-GAs are molecules with 20 carbon atoms, whereas the C_{19}-GAs have specifically lost the C-20 group and carry instead a γ–lactone ring (Figure 2a and b). In addition to free GAs, plants contain several GA conjugates, including GA-*O*-β-glucosides and β–glucosyl ethers (see Crozier *et al.,* 2001).

The discovery of the cytokinins occurred in 1955, when F. Skoog isolated a substance called kinetin from an autoclaved sample of DNA and demonstrated it to be active *in vitro* in promoting mitosis and cell division in tobacco callus tissues (see Moore, 1979). Although kinetin is an artefact derived from 2-deoxyadenylate, its biological activity resembles that of zeatin (Z) (Figure 2c), a native inducer of

plant cell division that was isolated from immature maize seeds in 1963 (see Crozier *et al.,* 2001).

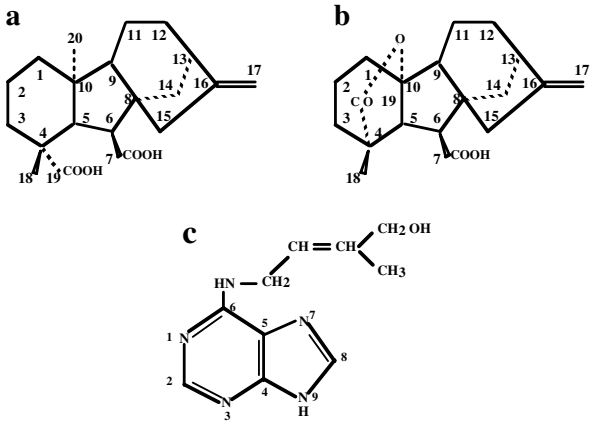

Figure 2. Basic structure of C_{20} (a) and C_{19} (b) gibberellins and the chemical structure of zeatin(c). The numbering of the ring systems of GAs derives form the nomenclature used for diterpenes, modifications at positions 2, 3 and 20 are important for biological activity. Cytokinins have the same general structure as zeatin, with different branched carbon substituents at positions 6, 2, 7 and 9.

Ethylene, which is recognized as “the ripening hormone”, was identified some 50-plus years ago (Burg, 1962). Many soil bacteria have genes that code for aminocyclopropane deaminase (AAC-deaminase), which degrades a key intermediate in ethylene production, thus preventing ethylene accumulation by plants (Penrose and Glick, 2003). Abscissic acid was discovered around 1960 as the hormone causing abscission of fruits and dormancy of buds (see Moore, 1979).

2. PRODUCTION AND ROLE OF PHYTOHORMONES

It is now well established that there are two sources of phytohormones naturally available for the plants: endogenous production by the plant tissues and exogenous production by associated microorganisms, including numerous soil bacteria and fungi (see Kumar and Lonsane, 1989; Arshad and Frankenberger, 1991; Costacurta and Vanderleyden, 1995; Patten and Glick, 1996).

2.1. Diversity of the Plant Hormone Producers

The ability to synthesize IAA, GAs, and cytokinins is widespread among soil and plant-associated bacteria and are responsible for plant-growth promotion, symbiotic associations, and also pathogenesis.

Examples of phytohormone producers are reported in Table 1 as well as the effect on the plant physiology and development. It appears that bacterial production of IAA and cytokinins is involved in the virulence of several interactions involving microorganisms, such as *Agrobacterium, Pseudomonas savastanoi* and pathogenic *Erwinia* (Comai and Kosuge, 1982; Costacurta and Vanderleyden, 1995; Litchter *et al.,* 1995; Morris, 1986). In contrast, in other bacteria, such as members of the genera *Azospirillum*, *Rhizobium*, *Bradyrhizobium*, *Enterobacter*, *Erwinia* and other *Pseudomonas* spp., production of phytohormones may be beneficial by stimulating plant growth (Patten and Glick, 1996). Additional information relative to ethylene production and other hormones can be found in section 6.

Table 1. Phytohormones produced by plants and microorganisms and their effect on plant morphology and development

Auxins production

Plant endogenous production or causative agent	Observed effect on plant	Reference
Plant		
Zea mays	cell enlargement,	Östin *et al.,* 1999
Arabidopsis thaliana	root initiation,	Bartel, 1997
	vascular differentiation,	Bartling *et al.,* 1994
	apical dominance	
Fungus		
Pisolithus tinctorius	plant growth promotion	Frankenberger and Poth,1987
Bacterium		
Azospirillum	decrease of root length,	Tien *et al*., 1979
Rhizobium,	increase of root-hair	Atzorn *et al*.,1988
Bradyrhizobium	development,	Badenosch-Jones *et al*., 1982
Klebsiella	increase in root branching and root surface	El-Kawas and Adachi, 1999
Azospirillum,	inoculated corn seedlings	Fuentes-Ramírez *et al*., 1993
Gluconacetobacter	showed an increase on free	Bastián *et al.,* 1998
Herbaspirillum	active IAA, and IBA	Fallik, *et al.,* 1989
Pseudomonas syringae pv *savastanoi*	induction of gall and tumor formation	Comai and Kosuge, 1980; 1982
Agrobacterium		Liu *et al.,* 1982
Erwinia herbicola pv *gypsophilae*		Manulis *et al*.,1998
Cyanobacteria, *Nostoc*	symbiotic tissue of *Gunnera*	Sergeeva *et al*., 2002

Gibberellins production

Plant endogenous production or causative agent	Observed effect on plant	Reference
Plant		
Arabidopsis thaliana	seed germination, development and reproduction of plants, floral development	Kobayashi *et al*., 1994
Oryza sativa		Helliwell *et al*., 2001
Zea mays		Spray *et al.,* 1996
Pisum sativum		
Fungus		
Gibberella fujikuroi	"bakanae" effect in maize, rice, and other plants	Rojas *et al*., 2001 Fernández-Martin *et al.,* 1995
Bacterium		
Azospirillum brasilense *Azospirillum lipoferum*	reversion of dwarfism in maize and rice	Cassán *et al*., 2001
Azospirillum brasilense	promotion of shoot elongation, growth, and root-hair density	Fulchieri *et al., 1993*

Cytokinins production

Plant endogenous production causative agent	Observed effect on plant	Reference
Plant		
Arabidopsis thaliana	cell division, chloroplast differentiation, photosynthesis, senescence, and nutrient metabolism	Takei *et al.,* 2001
Bacterium		
Azospirillum	Plant-growth promotion	Tien *et al.,* 1979
Pseudomonas syringae pv *savastanoi* *Agrobacterium tumefaciens*	induction of gall and tumor formation	Roberto and Kosuge, 1987
Erwinia herbicola		Lichter *et al.,* 1995

2.2. Effect and Role of Plant Hormones on the Plant Physiology and Development.

Plants have evolved elaborated systems for regulating cellular levels of IAA. Homeostatic regulation of the size of the free IAA pool is the result of different processes, including synthesis, degradation, conjugation (with either amino acids or sugars), and transport (Normanly and Bartel, 1999).

IAA represents one of the most important plant hormones. It regulates many aspects of plant growth and development throughout the plant cell cycle, from cell division, cell elongation and differentiation to root initiation, apical dominance, tropistic responses, flowering, fruit ripening, and senescence. Regulation of these processes by auxin is believed to involve auxin-induced changes in gene expression (Guilfoyle *et al.,* 1998). There is no complete description of the mechanism by

which auxin regulates cell growth. The immediate effect of exposure of plants tissues to auxin is proton excretion, occurring within minutes. The resulting apoplastic acidification provides a favourable condition for cell-wall loosening, which could be an early part of auxin-induced cell expansion (Kim *et al.,* 2001).

Auxin-binding proteins (ABPs) are a class of low abundance proteins in plants that bind active auxins with high affinity and specificity; thus, most likely acting as plant receptors for the auxin signal. As a result of ABP-auxin binding, ABP might initiate the auxin-signalling pathway leading to various cellular responses. Extensive studies have led to the identification of a number of ABPs in both membrane and soluble fractions, the best characterized of which (in terms of cellular localization, biochemical nature, and putative receptor function) is ABP_1 (Kim *et al.,* 2001).

In conjunction with auxins, cytokinins promote cell division. They also influence differentiation of plants cells in culture. In plant-tissue culture (*in vitro*), a high cytokinin/auxin ratio promotes shoot production whereas auxin alone initiates root growth. Approximately equimolar amounts of cytokinin and auxin cause largely undifferentiated callus cells to proliferate. Cytokinins are involved in processes such as photosynthesis or chloroplast differentiation. They also are known to induce the opening of stomata, to suppress auxin-induced apical dominance, and to inhibit senescence of plants organs, especially in leaves (Crozier *et al.,* 2001).

Although best known for their influence on stem elongation, GAs also affect reproductive processes in a wide range of plants. In some plants species, exposure to low temperatures can induce seed germination or flowering. GAs are implicated in these processes, known respectively as stratification and vernalization. GAs retard leaf and fruit senescence and also induce de novo synthesis of alpha-amylase and other enzymes in the aleurone layer of barley. In dwarf varieties of rice, such as Tanginbozu, doses of GA control shoot elongation (Crozier *et al.,* 2001).

3. PATHWAYS FOR PLANT HORMONE BIOSYNTHESIS: COMMON ROUTES IN PLANTS, BACTERIA AND FUNGI

The early discovery of auxins and of their important role in plant development has generated considerable interest and elucidation of the biosynthetic routes for IAA and other plant hormones in bacteria largely depends on knowledge accumulated in plants and, in the case of gibberellins, in fungi.

3.1. Indole-3-Acetic Acid Synthesis

IAA is a simple metabolite that is derived from tryptophan (Trp) by multiple enzymatic pathways, but it can also by synthesized by Trp-independent routes, especially in plants (Figure 3). IAA biosynthesis may proceed by one or more pathways in both plants and bacteria. In addition, in plants, several genes (such as a gene family) may encode a particular enzyme within a pathway. This section emphasizes the routes established in plants. The situation for phytopathogenic bacteria and *Azospirillum* is further detailed in sections 4 and 5.

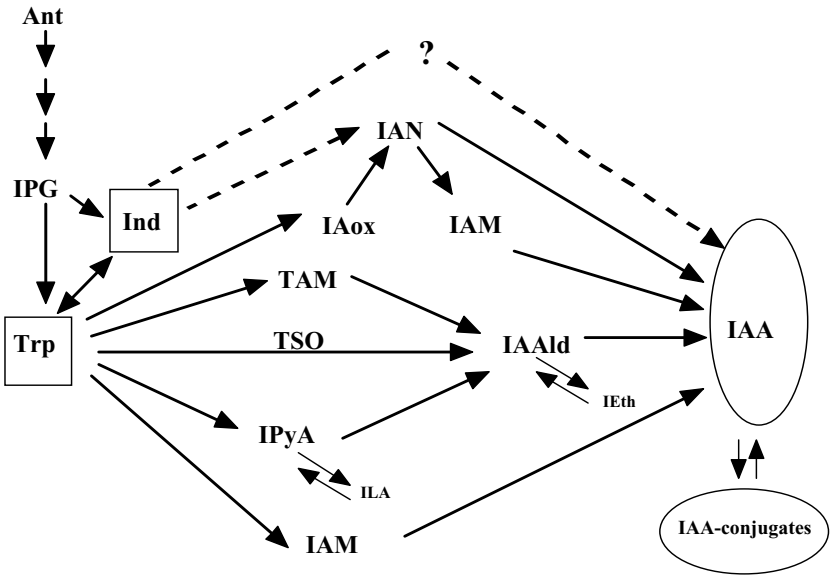

Figure 3. Biosynthetic pathways for IAA (indole-3-acetic acid) found in plants and bacteria. Trp-dependent routes: the IPyA (indole-3-pyruvate) and TAM (tryptamine) routes are found in both plants and bacteria; the IAM (indole-3-acetamide) route is specific to bacteria and plants transformed by Agrobacterium *T-DNA; the IAN (indole-3-acetonitrile) route (via IAox, indole-3-acetaldoxime) is found in plants, whereas in bacteria, IAN can be converted directly to IAA or via IAM, but the precursor of IAN may or not be Trp; the TSO (Trp side chain oxidation) route is specific of some* Pseudomonas*. Trp-independent routes, which are indicated as dotted lines, starting from Ind (indole) or IPG (indole-3-glycerol phosphate) may constitute major routes for IAA synthesis in plants, and may be present in bacteria. Enzymes involved in IAA-conjugate synthesis and degradation are found in both plants and bacteria. In all cases, anthranilate (Ant) is the precursor of the indole moiety. Ind can be converted to IPG, ILA (indole-3-lactic acid) can be converted to IPyA, and IEth (indole-3-ethanol) can be converted to IAAld (indole-3-acetaldehyde).*
See text for additional explanations.
Adapted from Costacurta and Vanderleyden, 1995; Patten and Glick, 1996; Normanly et al.*, 1999; Carreño-Lopez* et al.*, 2000.*

3.1.1. Tryptophan-Dependent Pathways for Indole-3-Acetic Acid Synthesis

The indole-3-pyruvic pathway (IPyA), which is common in higher plants (Normanly *et al.* 1995), is found in numerous soil bacteria (Brandl *et al.,* 1996; Costacurta *et al*., 1994; Koga *et al*., 1991b; detailed in sections 4 and 5).

Trp → IPyA → indole-3-acetaldehyde (IAAld) → IAA

A *ROOTY* gene, which encodes a protein similar to a tyrosine aminotransferase (for the first step of this pathway), has been isolated in *Arabidopsis*, but its role in

the production and control of auxin has not be elucidated yet (Gopalraj *et al.*, 1996). The indole-3-pyruvate decarboxylase (for the second step) has been purified from *E. cloacae*, but not from plants (Koga, 1995; see section 4). In contrast, aldehyde oxidase activities that catalyze the oxidation of IAAld to form IAA (in the third step) were found in *Arabidopsis* (Seo *et al.,* 1998).

The indole-3-acetamide pathway (IAM), which occurs in all gall-forming bacteria, was also described in *Bradyrhizobium japonicum* and *Rhizobium fredii* (Sekine *et al.,* 1989). This pathway is unique to bacteria and is only detected in plants infected with the pathogenic bacteria or in plant cells transformed by insertion of an *Agrobacterium* T-DNA (see section 4).

Trp $\rightarrow$ IAM $\rightarrow$ IAA

The indole-3-acetonitrile (IAN) pathway is another Trp-dependent route that has been characterized both in plant and bacteria. The nitrile, which is generated from IAox that occurs naturally in plants, is converted to IAA by nitrilases (Hull *et al*., 2000; Mikkelsen *et al.,* 2000). Four genes that encode nitrilases and are responsible for the conversion of IAN to IAA have been cloned from *Arabidopsis* (Bartling *et al*., 1994). However, only the nitrilase genes, *NIT1* and *NIT2*, have been shown to be involved in IAA biosynthesis *in vivo* (see Normanly and Bartel, 1999).

Trp $\rightarrow$ indole-3-acetaldoxime (IAox) $\rightarrow$ IAN $\rightarrow$ IAA

The discovery in *Arabidopsis* of two cytochrome P450 enzymes (CYP79B2 and CYT79B3), which catalyze the formation of IAox from Trp, suggests that at least a portion of IAN could be Trp-derived *via* an IAox intermediate. IAN may also be regarded as a degradation product that results from the turnover of indole glucosinolates. The enzymatic activity of CYP79B2, assayed in *E. coli* carrying a recombinant plasmid, showed that this cytochrome is specific for Trp (Hull *et al*., 2000). The cloning of *CYP79B2* from *Arabidopsis* was also accomplished by Mikkelsen *et al*. (2000). The gene encodes a 61-kDa polypeptide with 85% amino-acid identity to *CYP79B3*. The conversion of Trp to IAox by the recombinant CYT79B3 permitted the chemical identification of this latter compound. It was proposed that cross-talk might occur between the biosynthetic pathways of indole glucosinolates and IAA at the IAox branch point. Support for this hypothesis came from Barlier *et al*., (2000), who showed that a mutation in the gene encoding the CYP83B1 protein, which belongs to the family of cytochrome P450, induces auxin over-production. This led to elevated IAA levels and thus to both increased apical dominance and reduced indole glucosinolate levels. Conversely, over-expression of CYP83B1 in *Arabidopsis* led to a reduced IAA level and loss of apical dominance, which correlated with an elevated indole glucosinolate level. Here, the increased N-hydroxylation of IAox results in a net loss of IAA (Bak *et al.,* 2001).

Another pathway involving tryptamine (TAM) may be common to both plants and bacteria, particularly to members of the *Azospirillum* genus (see section 5). Using an *Arabidopsis* mutant that had an elevated level of endogenous auxin production, a flavin monooxygenase (FMO)-like enzyme, which catalyzes the hydroxylation of TAM, was identified (Zhao *et al.,* 2001).

3.1.2. Trp-Independent Pathway for IAA Synthesis

Work with Trp auxotrophs and quantitative measurements with labeled products both in plants and bacteria have established that IAA biosynthesis can also take place *via* a Trp-independent route. In some plants, the Trp-independent pathway is thought to be the primary route for IAA production. The likely precursors for the "Trp-independent" pathway are indole-3-glycerol phosphate (IPG) or indole.

In maize, the occurrence of an IAA-biosynthetic pathway that does not use Trp as an intermediate was confirmed by experiments based on either [^{15}N]anthranilic acid or $^{2}H_2O$ labeling of *orange pericarp* seedlings, which showed incorporation of radioactivity into IAA but not into Trp (Wright *et al*., 1991). Light-grown seedlings of normal maize and the maize mutant *orange pericarp* were shown to contain enzymatic activity able to convert [^{14}C]indole to IAA. Trp did not inhibit the reaction and neither [^{14}C]Trp nor [^{14}C]serine could replace [^{14}C]indole. The formation of IAA was proved by gas chromatography-mass spectrometry (GC-MS) analysis (Östin *et al*., 1999).

The isolation of Trp auxotrophs in *A. thaliana* helped to determine whether or not Trp is the sole precursor to IAA. The levels of free IAA did not differ significantly between the wild type and the *trp1-1* mutant that was defective in anthranilate phosphoribosyltransferase activity. In contrast, the *trp2-1* mutant, which was deficient in tryptophan synthase activity, showed an elevated level of IAA that correlated with a dramatic increase in indole production. *In vitro* labeling experiments with *trp2-1* seedlings grown in the presence of both [$^{2}H_5$]Trp and [^{15}N]anthranilate led to the conclusion that IAA biosynthesis occurs *via* a Trp-independent pathway (Normanly *et al*., 1993).

Using a different approach, Ouyang *et al* (2000) suggested that indole might be a precursor for the Trp-independent pathway and that indole-3-glycerol phosphate (IGP) played a critical role. Their strategy was based on the use of available mutants from *Arabidopsis*, including mutants defective in anthranilate synthetase α and β (TSA and B), anthranilate phosphoribosyltransferase, and tryptophan synthase. The Trp pool in these mutants was decreased as compared to the wild-type. Because no mutant defective in indole-glycerol phosphate synthetase (IGS) was available, Ouyang *et al.* (2000) constructed transgenic plants harboring antisense *IGS* RNA. Total levels of IAA were significantly decreased in *IGS* transgenic plants, whereas the IAA pool increased in plants mutated in the tryptophan synthase gene. These results suggested that IGP is the branch point of the Trp-independent IAA synthesis in *A. thaliana*.

In spite of data described above, the Trp-independent pathways remain poorly defined in terms of the enzymes, their intermediates, and cellular localizations (Östin *et al.,* 1999).

3.2. Gibberellins

The GAs are complex molecules of tetracarbocyclic diterpernes. *G. fujikuroi* synthesizes about 20 different gibberellins of which the most abundant is the gibberellic acid (GA_3) (Fernández-Martín *et al*., 1996). About 100 have been exclusively isolated from plants. The numbering used with GAs is not related to their structure. Those molecules, whose structure has been elucidated, are numbered in the approximate order of their discovery.

There is continuing interest in the biosynthetic origin of the GAs because some of them have important activities in plants. The most important GA in plants is GA_1, which is primarily responsible for stem elongation. In *Gibberella,* GA biosynthesis is catalyzed by enzymes falling into three classes: terpene cyclases that catalyze the synthesis of *ent*-kaurene from geranylgeranyl diphosphate; cytochrome P450 monooxygenases that catalyze the steps of the pathway from *ent*-kaurene to GA_{12}; and soluble dioxygenases that catalyze the final steps of the pathway (Helliwell *et al.,* 2001). Early steps are identical for both plants and fungi (Figure 4), but the pathways diverge thereafter and at least three different routes are known. The studies performed in plants also showed the presence of the early-13-hydroxylation pathway, which is unique in plants (Spray *et al.,* 1996).

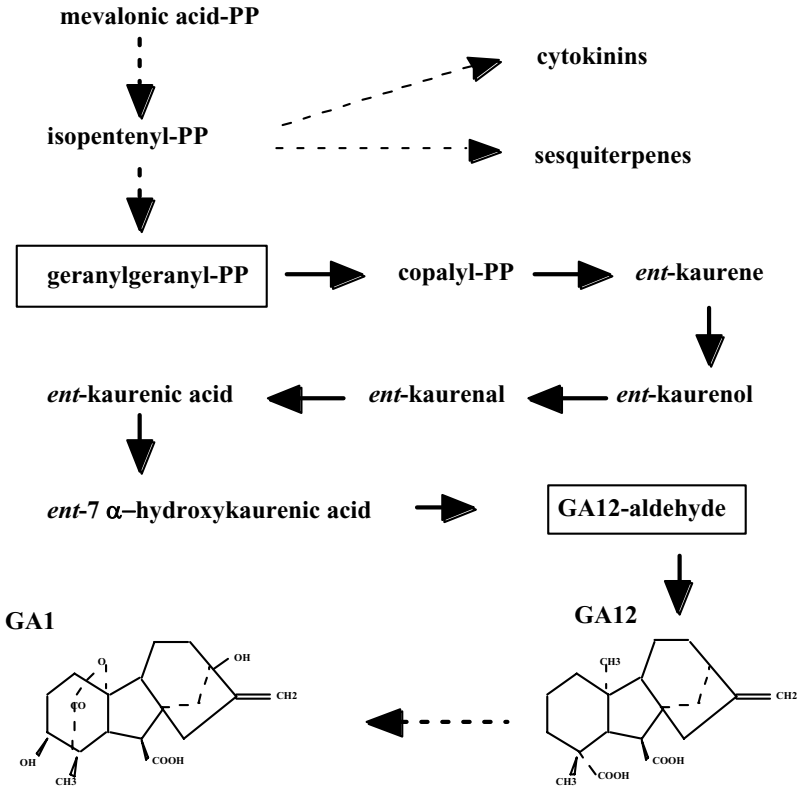

Figure 4. Schematic biosynthetic pathways of GAs.
GAs are biosynthesized from trans-*geranyl diphosphate via* ent-*copalyl diphosphate and the tetracyclic hydrocarbon* ent-*kaurene.* ent-*kaurene is sequentially oxidized to* ent-*7α–hydroxykaurenoic acid, which is then arranged to GA_{12}-aldehyde, oxidized to GA_{12}, and metabolised to other GAs. The figure also shows branching from isopentenyl-PP to cytokinins and sesquiterpenes (abscisic acid).*

3.3. Cytokinins

Cytokinins are adenine derivatives. Studies with the slime mold *Dictyostelium discoideum* revealed that 5'-AMP was a direct precursor of isopentenyl adenosine 5′-phosphate ([9R-5'P]iP). The enzyme catalyzing this conversion, dimethylallyl diphosphate: 5'-AMP transferase (or isopentenyl transferase), was also found in cell-free extracts from maize kernels, and from tobacco callus tissue cultures that became cytokinin-autonomous (Crozier *et al.*, 2001). Recently, several genes encoding the isopentenyl transferase have been identified from *A. thaliana* (Takei *et al.,* 2001). A corresponding enzyme from the bacterium *A. tumefaciens*, encoded by the *ipt* gene, has been studied in depth at the molecular level, and the same gene was also found in *Pseudomonas syringae* pv. *savastanoi*, where it is named *ptz.* These genes encode for products that share substantial sequence similarity and both are involved in tumor-inducing ability. However, tumor induction by *Pseudomonas* does not involve transfer of genetic material to the plant genome as shown with *A. tumefaciens* (Roberto and Kosuge, 1987).

3.4. Ethylene

Ethylene biosynthesis by plants originates from methionine. The first step is the synthesis of S-adenosyl-methionine, followed by its conversion into 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is the direct precursor of ethylene. ACC oxidase, formerly known as the ethylene-forming enzyme (EFE), was first characterized in apple (Adams and Yang, 1979). Ethylene production has been also reported for bacteria and fungi (Arshad and Frankenberger, 1991; Fukuda *et al*., 1993; see section 6.3).

4. MAJOR ROUTES FOR IAA SYNTHESIS IN PATHOGENIC AND BENEFICIAL NITROGEN-FIXING BACTERIA ASSOCIATED WITH PLANTS

4.1. Discovery and Conditions of Synthesis

During the past 25 years, the standard techniques used in natural product chemistry have been extended to phytohormone research. Physico-chemical methods, such as high performance liquid chromatography (HPLC) and GC-MS, have been developed for the identification and measurement of hormones. The accuracy and facility of quantitative measurements have been further improved by the use of labeled substrates.

Early reports with *Azospirillum brasilense* showed that it produced less than 2µg/ml IAA in N-free medium but up to 24µg/ml IAA in NH_4-containing medium supplemented with Trp (Tien *et al.,* 1979). Several strains of *A. brasilense* and *Azospirillum lipoferum*, isolated from maize and teosinte, produced IAA and related indoles, such as ILA and IEth. The amounts of IAA obtained depended on the species and strain as well as on the conditions of their cultivation, including the presence of Trp, oxygenation level, pH, and growth phase (Crozier *et al.,* 1988). Addition of Trp to culture media of an *A. brasilense* strain strongly stimulated the

release of IAA, which showed a rise at the stationary phase (Omay *et al.,* 1993). This result supports the existence of a Trp-dependent route for IAA biosynthesis. The *A. brasilense* strain UAP154, isolated from maize, produced IAA as well as IBA, both of which were identified by HPLC and GC-MS (Martínez-Morales, *et al.,* 2003).

Physiological studies with *Enterobacter cloacae* and the epiphytic strain *Erwinia herbicola* showed that Trp, IPyA, and IAAld were transformed into IAA (Koga *et al.,* 1991a). There was no indication of the existence of the IAM pathway in these bacteria. Therefore, it was concluded that the IPyA pathway could be the primary route for IAA biosynthesis in *E. cloacae* as well as in *E. herbicola* 299R (Brandl *et al.,* 1996; Koga *et al.,* 1991a).

IAA has been detected in culture supernatants of *Rhizobium*. Similar concentrations were found in the supernatants of wild-type strains and corresponding *nod*-mutants (Badenoch-Jones *et al*., 1982). Metabolic studies with ^{3}H-, ^{14}C-, and ^{2}H-labeled substrates demonstrated that *Rhizobium leguminosarum* biovar *phaseoli* was able to convert: (i) Trp to IAA, IEth, and indole-3-methanol (IM); (ii) IEth to IAA, and IM; and (iii) IAA to IM. Because the conversion of IEth to IAAld is a reversible reaction, a storage role for IEth in IAA biosynthesis was proposed. IAM was detected neither as an endogenous constituent nor as a metabolite of ^{3}H-Trp, and cultures did not convert IAM to ^{14}C-IAA (Ernstsen *et al*., 1987).

Fuentes-Ramírez *et al* (1993), using a chemically defined culture medium, characterized both IAA and IAA-conjugates in supernatants of *Gluconacetobacter diazotrophicus* cultures. However, only IAA was detected in supernatant cultures from *Herbaspirillum seropedicae* (Bastián *et al*., 1998).

4. 2. The IAM Pathway in Plant Pathogens

The IAM pathway has been studied in detail in *A. tumefaciens* and *P. syringae* pv. *savastanoi*. These two phytopathogens have drawn much attention in attempts to understand the role of phytohormone in virulence. IAA is produced from Trp by sequential action of two enzymes, Trp 2-monooxygenase and indole-3-acetamide hydrolase, which catalyze the conversion of Trp to IAM and IAM to IAA, respectively (Follin *et al.,* 1985; Hutcheson and Kosuge, 1985; Van Onckelen *et al*., 1986; Thomashow *et al*., 1984; Schröder *et al.,* 1984). These two phytopathogens have been the focus of much attention on understanding the role of phytohormone production in virulence.

A. tumefaciens can infect a wound site of dicotyledonous plants and cause the formation of crown gall tumors. Virulent strains of *A. tumefaciens* contain large plasmids, called pTi (tumor inducing). During the course of infection, a portion of the pTi, the T-DNA, is stably transferred to the plant cells where it becomes integrated into the nuclear genome. Expression of specific genes encoded by the T-DNA causes an alteration in the normal metabolism of auxins and cytokinins (Morris, 1986). The structural genes encoding the IAM hydrolase and the Trp 2-monooxygenase, *tms-1*and *tms-2,* are part of the T-DNA (Inzé *et al*., 1984; Thomashow *et al.,* 1984; Schröder *et al.,* 1984).

These two genes have their counterparts in *P. syringae* pv. *savastanoi,* namely *iaaM*, and *iaaH* genes (Comai and Kosuge, 1982). The bacterial pathogen *P. syringae* pv. *savastanoi* invades the tissue of oleander, olive and privet, where it induces tumorous overgrowths called galls. Tumor formation by these plants is a response to the high concentration of IAA produced by the bacteria. Loss of the capacity to produce IAA was correlated with a loss of a plasmid (pIAA1) that carried the *iaaM* and *iaaH* genes and controlled IAA production and virulence (Comai and Kosuge, 1980). The *iaaM* and *iaaH* genes are organized in an operon, whereas the comparable genes in T-DNA are monocistronic (Yamada *et al*., 1985). However, the *tms* genes from *A. tumefaciens* are not biologically active in the bacteria and exert their pathogenic influence only within the plant-cell environment.

P. syringae pv. *savastanoi* strains isolated from oleander can convert IAA to a conjugate form, indoleacetyl-ε-lysine (IAA-Lys), although they do not produce IAA-lysine in the free-living state. The genetic determinant (*iaaL*) for the enzyme is located on pIAA1, but is not part of the *iaa* operon (Glass and Kosuge, 1986). An *iaaL*-Tn*5* mutant from an oleander-gall isolate did not convert IAA to IAA-Lys. Further, although it accumulated five-fold more IAA in free-culture, this mutant did not cause typical gall symptoms and it did not proliferated within host tissues as well as the wild-type strain. Because free IAA is susceptible to degradation by host-plant peroxidases but IAA conjugates are resistant to degradation, it was presumed that IAA (being the active form in promoting gall formation) is released in plant tissues as a result of hydrolysis of IAA-lysine. Then, it was suggested that expression of *iaaL* helps modulate the IAA concentration in plants (Glass and Kosuge, 1988).

The survey of a large number of *P. syringae* strains, belonging 57 different pathovars, for IAA production and presence of *iaaH* and *iaaM* genes revealed a large heterogeneity (Gardan *et al*., 1992; Glickmann *et al*., 1998). Most of the strains produced IAA after growth in Trp-containing medium. Surprisingly, *iaaH* and *iaaM* were detected in a limited number of strains, suggesting that IAA synthesis in most pathovars of *P. syringae* does not proceed through the IAM pathway. Instead, they may possess the IPyA route (Glickmann *et al.*, 1998). Some of the assayed strains produced high concentration of IAA even in the absence of added Trp. Those isolates are good candidates for investigating IAA synthesis *via* a Trp-independent route, even though they carried both the *iaaH* and *iaaM* genes. Interestingly, most of the strains contained an *iaaL* gene (Glickmann *et al.*, 1998). This observation supports the regulatory role of IAA-lysine synthetase in modulating the IAA concentration and indicates that IAA-amide conjugate synthesis is not specific to bacteria having the IAM pathway.

4.3. Discovery of the IPyA Pathway

The first step involved in IPyA pathway is the conversion of L-Trp to IPyA catalyzed by aromatic aminotransferases (AAT). Multiple proteins with AAT activity have been identified on non-denaturing polyacrylamide gels of crude extracts (Lewis-Kittell *et al.,* 1989; Baca *et al.,* 1994). These enzymes are common in bacteria and they are non-specific with respect to their aromatic amino-acid

substrates (Koga *et al.,* 1994; Pérez-Galdona et *al.,* 1992; Soto-Urzúa *et al.,* 1996). The *E. cloacae* AAT displays a high K_m value for Trp whereas the K_m for IPyA is 138-fold lower (Koga *et al.,* 1994). Moreover, IPyA is a competitive inhibitor of the reaction responsible of its own production in *Azospirillum* (Soto-Urzúa *et al.,* 1996). However, the affinity for IPyA of the second enzyme in the pathway is very high (see below) and favours the net synthesis of IAA (Koga, 1995). Genetic evidence for the involvement of AAT1 and AAT2 in IAA production was obtained in *Sinorhizobium meliloti*. Both enzymes contributed to IAA biosynthesis when a high level of Trp was presented (Lewis-Kittell *et al.,* 1989).

The second step of the pathway, first discovered in *E. cloacae,* is the conversion of IPyA to IAAld and is catalyzed by a novel enzyme, the indolepyruvate decarboxylase (IPDC) (Koga *et al.,* 1991b; 1992). The function of the *ipdC* gene was established by the conversion IPyA to IAAld by a cell-free system prepared from *E. coli* harbouring *ipdC* on a recombinant plasmid (Koga *et al.,* 1991b). The crystal structure of this enzyme has been determined (Schutz *et al*., 2003). Its predicted amino-acid sequence shows extensive homology with that of pyruvate decarboxylase enzymes, which catalyze the decarboxylation of pyruvate to acetaldehyde and CO_2. The IPDC enzyme is a homotetramer, which uses both thiamine pyrophosphate and Mg^{2+} as cofactors. It has a much higher affinity for IPyA, with a K_m value for IPyA of 15µM, than for pyruvic acid. These results indicate that IPDC from *E. cloacae* is a highly specific enzyme with a high affinity for IPyA (Koga *et al.,* 1992).

Zimmer *et al.* (1994) found the *ipdC* gene in several *Enterobacteriaceae* by PCR amplification. Further genetic evidence for the role of IPDC in biosynthesis of IAA came from the studies performed in *A. brasilense* Sp245 and Sp7 strains (Costacurta *et al*., 1994; Zimmer *et al.,* 1998b; Carreño-Lopez *et al*., 2000), *A. lipoferum* (Yagi *et al.,* 2001), *E. herbicola* (Brandl and Lindow, 1996), and *Pseudomonas putida* (Patten and Glick, 2002a). Indeed, the loss of the ability of all *ipdC* mutants to synthesize IAAld is consistent with the conclusion that the *ipdC* gene codes for an IPDC activity, a part of the IAA pathway, in these bacteria. Expression of *ipdC* from *E. herbicola* 299R and *P. putida* GR12-2 was monitored using transcriptional fusions. With *E. herbicola*, the *ipdC* gene was expressed at low levels in culture medium, where expression was independent of pH, fixed-nitrogen and Trp availability, O_2, and the growth phase of the culture (Brandl and Lindow, 1997). In contrast, Trp induced *ipdC*-gene expression in *P. putida* GR12-2 and its transcription was regulated by the stationary-phase sigma factor, RpoS (Patten and Glick, 2002b).

4.4. The IAN Pathway

Although the production of IAN in bacteria has not been fully investigated, evidence for the IAN pathway in *Agrobacterium*, *Rhizobium* (Kobayashi *et al.,* 1995), and *Azospirillum* (Carreño-Lopez *et al.,* 2000) has been reported. Microbial degradation of IAN can proceed *via* two routes: (i) using a nitrilase that catalyzes the direct conversion of nitriles into the corresponding acid plus ammonia; and/or (ii) using a nitrile hydratase that catalyzes the conversion of IAN to IAM, which is

then converted to IAA and ammonia by an amidase. The occurrence of both nitrile hydratase and amidase activities was detected in several strains of *Agrobacterium*, *R. leguminosarum*, *R. loti*, and *S. meliloti*. A nitrile hydratase from *A. tumefaciens* was characterized as a homotetramer, with a K_m of 7.9 μM for IAN (Kobayashi *et al.,* 1995).

4.5. Other Pathways

Early work performed in *Pseudomonas fluorescens* revealed another pathway involved in IAA biosynthesis, the "tryptophan side chain oxidase" (TSO), which is able to convert Trp to IAAld. TSO is an inducible pathway that reaches its maximal activity at the stationary phase (Narumiya *et al*., 1979; Oberhänsli *et al.,* 1991).

5. MULTIPLE ROUTES FOR IAA SYNTHESIS IN *AZOSPIRILLUM*

IAA production has been investigated in *Azospirillum*. Although conflicting data were reported, several biosynthetic pathways are present in this genus. However, differences exist between *Azospirillum* species and probably within strains of the same species.

5.1 Evidence for Multiple Pathways

Trp is generally considered as the IAA precursor in *Azospirillum* (Crozier *et al*., 1988; Baca *et al*., 1994). Because none of the mutants impaired in IAA synthesis was totally unable to produce IAA, *Azospirillum* likely contains several routes for IAA synthesis (Hartmann *et al.* 1983; Barbieri *et al.,* 1986; Ruckdäschel and Klingmüller, 1992; Gastélum-Reynoso *et al*., 1994).

Prinsen *et al*. (1993) provided evidence for at least three different routes based on the use of ^{3}H-Trp and ^{3}H-IAM. With the wild type, if ^{3}H-Trp was added to a bacterial culture, only 10% of the IAA was found to be radioactive, suggesting the existence of a Trp-independent route. When ^{3}H-IAM was added to culture, only very low specific radioactivity was incorporated to IAA (0.1%), suggesting that a Trp-dependent pathway, which was different from the IAM pathway, existed in *Azospirillum*. In addition, using a low IAA-producing Tn*5*-induced mutant, an increased accumulation of IAM from radioactive Trp was found. This finding supported the existence of an unidentified Trp-independent pathway responsible for most, if not all, IAA production, when the bacteria are placed in Trp-limiting conditions. Thus, *Azospirillum* has this one Trp-independent route plus two Trp-dependent routes, one of them likely being the IAM route.

In agreement, physiological studies performed with the intermediate IAM, along with hybridization experiments, led to assessment that the IAM pathway was present in *A. brasilense* (Bar and Okon, 1993), even though Zimmer *et al.* (1991) had failed to demonstrate the presence of this pathway. Thus, in addition to multiple routes, different pathways may exist in different strains.

5.2. Biochemical and Genetic Evidence for the IPyA Route

The initial reaction, conversion of Trp into IPyA, can be catalyzed by aromatic aminotransferases, and several of them have been reported in *A. brasilense* and *A. lipoferum* (Ruckdäschel *et al.,* 1988; Baca *et al.,* 1994). The IPyA route in *Azospirillum* was demonstrated through the genetic characterization of a low IAA-producing mutant. Complementation studies with an *A. brasilense* strain Sp245 library led to a gene encoding a protein with extensive homology with IPDC of *E. cloacae* (Costacurta *et al.,* 1994). Thereafter, the *ipdC* gene was isolated from *A. brasilense* Sp7 and *A. lipoferum* FS. Knockout mutants were found to synthesize about 10% of the level of IAA produced by wild type, indicating that the IPDC enzyme is a key enzyme for IAA biosynthesis in these bacteria (Costacurta *et al.,* 1994; Zimmer *et al.,* 1998b; Carreño-Lopez *et al.,* 2000; Yagi *et al.,* 2001).

Regulation studies determined that *ipdC* is expressed in the late exponential phase of growth, depending on cell density, but independently of the presence of Trp (Vande Broek *et al.,* 1999; Carreño-Lopez *et al.,* 2000). Vande Broek *et al* (1999) also reported that *ipdC* expression is upregulated by IAA, a finding not observed by Carreño-Lopez *et al.* (2000). However, an element, which resembles the auxin-responsive element "Aux-RE", was found upstream of the *ipdC* gene (Lambrecht *et al*., 2000). In addition, inoculation of wheat roots with an *Azospirillum* Sp7 derivative that carried a chromosomal *ipdC-lacZ* transcriptional fusion, revealed a significant expression of *ipdC,* showing that the *ipdC* gene was expressed in association with the host plant (R. Carreño-Lopez, C. Elmerich and B. Baca, unpublished results). Upstream of the *ipdC* gene of *A. lipoferum* FS inverted repeat sequences (IRS) were found. Gel mobility-shift assays showed the presence of two DNA-binding proteins that might be involved in regulation of *ipdC*-gene expression. Further investigation is required to define the mechanism involved in regulation of *ipdC* by these proteins (Yagi *et al.,* 2001). To date, the only cloned gene involved in IAA biosynthesis is *ipdC.*

5.3. Alternative Trp-Dependent Routes in A. brasilense *Sp7: Physiological Evidence for the TAM and the IAN Pathways*

Identification of an alternative Trp-dependent route was derived from physiological experiments performed by Hartmann *et al.* (1983) and Carreño-Lopez *et al.* (2000). Mutants of *A. brasilense*, which were overproducing IAA, were observed to excrete a compound tentatively identified as TAM (Hartmann *et al.,* 1983). Later, it was observed that, although the IAA production of an *ipdC-Km* mutant strain was decreased significantly when bacteria were grown in either malate- or gluconate-containing media, its IAA production was similar to that of the wild-type when grown in media containing either lactate or pyruvate as carbon source. This observation strongly suggested that an alternative route, which was repressed in malate- or gluconate-containing media, compensated for this loss of the IPyA route in cells grown on lactate- or pyruvate-containing media (Carreño-Lopez *et al.,* 2000). Then, using a set of Trp auxotrophs, either with or without the *ipdC* mutation, the same authors showed that the alternative route was Trp dependent.

Indeed, a mutant that was unable to convert indole to Trp still produced IAA from Trp in lactate-containing medium, but did not produced IAA from indole.

Using permeabilized cells of both the wild type and the *ipdC-Km* mutant that were feed with different precursors of IAA, including TAM, IAN and IAM, it was subsequently observed that both TAM and IAN could be converted to IAA, whereas IAM could not (Carreño-Lopez *et al.,* 2000). Because the conversion of TAM was repressed by gluconate, it was concluded that TAM is a precursor of a Trp-dependent pathway, which was different from the IPyA pathway and which is subject to regulation by catabolic repression.

IAN was also found to be an intermediate for IAA biosynthesis, but in only minor proportions, and that IAN conversion was not fully repressed by gluconate (Carreño-Lopez *et al.,* 2000). IAM can be an intermediate in IAN conversion to IAA in other bacteria (Kobayashi *et al*., 1995). Thus, it is tempting to speculate that nitrile hydratase and amidase activities constitute an alternative pathway for IAA synthesis in *Azospirillum*. This reasoning could explain the IAM accumulation detected in the IAA low-producer mutant described by Prinsen *et al.,* (1993).

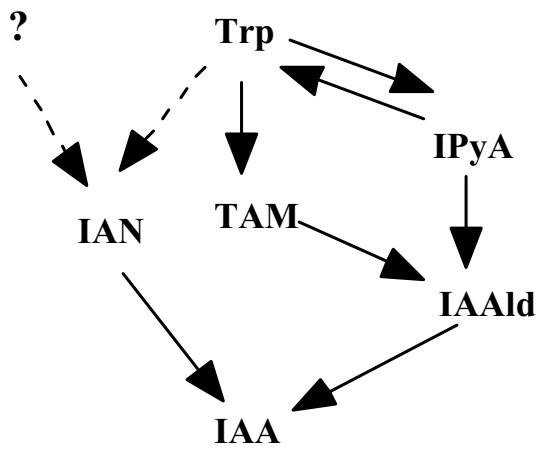

Figure 5. Routes for IAA synthesis in A. brasilense *Sp7.*
Abbreviations as in Figure 1. Adapted from Carreño-Lopez et al*. (2000).*

To conclude, *A. brasilense* appears to possess two differently regulated Trp-dependent routes for IAA synthesis, namely the IPyA and the TAM pathways, as well as an alternative route that uses IAN as an intermediate (Carreño-Lopez *et al*., 2000; see Figure 5). It remains to be established whether the IAN pathway is the Trp-independent route or if the route described by Prinsen *et al*. (1993) is a fourth, as yet, unidentified pathway.

5.4. Regulation of Trp Synthesis and IAA Production

Screening Trp-dependent IAA production of different *Azospirillum* species revealed that *A. irakense* KA3 released 10-times less IAA into the medium than *A. brasilense* Sp7. By genetic complementation with a cosmid library of strain Sp7, a DNA region that increased IAA production in *A. irakense* was identified as the *trpGDC* cluster, which is involved in Trp biosynthesis (Zimmer *et al*., 1991; Zimmer and Elmerich, 1992). Introduction into *A. irakense* of the *A. brasilense trpD* gene, which codes for the phosphoribosyl anthranilate transferase, resulted in decreased release of anthranilate (Ant) into the medium, due to the conversion of anthranilate by TrpD, with a concomitant increase in IAA production. It, thus, appears that the difference in Trp metabolism is correlated with IAA biosynthesis, and that *trpD* plays a role in the regulation of IAA biosynthesis. In agreement with this hypothesis, a mutant of *A. brasilense* Sp 245 that carries a Tn*5-mob* insertion in an 85 MDa plasmid differs from wild type in both enhanced Ant release and decreased IAA production (Katzy *et al.,* 1990). Therefore, it appears that Ant, which is an intermediate in Trp biosynthesis, probably represses IAA production in *A. brasilense*.

A. brasilense contains two anthranilate synthase activities. A *trpE* gene was isolated and found to encode a putative TrpE(G)-fusion protein (De Troch *et al.,* 1997). A putative leader and both terminator/anti-terminator loops were also identified. The formation of these latter structures is necessary for the regulation of the expression of the Trp operon by the peptide leader (De Troch *et al.,* 1997). In terms of Trp biosynthesis, the feedback inhibition of Ant synthase by Trp is one important regulatory mechanism that controls the cellular Trp pool. Indeed, mutants that excreted large amounts of IAA were isolated and shown to be altered in the feedback regulation of Ant synthetase by Trp (Hartmann *et al*., 1983). More recently, the *trpAB* genes that encode tryptophan synthase were cloned and sequenced (Dosselaere *et al.,* 2000).

6. OTHER PHYTOHORMONES PRODUCED BY PLANT PATHOGENIC AND NITROGEN-FIXING ASSOCIATED AND ENDOPHYTIC BACTERIA

6.1 Gibberellins

In their early work, Tien *et al.* (1979) detected gibberellin-like substances in supernatants from *A. brasilense* cultures at an estimated concentration of 0.05µg/ml GA_3 equivalent. GA_1 and GA_3 were also identified in cultures of the *A. lipoferum* op33 strain and a quantitative estimation, using the dwarf rice cv. Tan-ginbozu microdrop bioassay, showed that 20-40 pg/ml were produced (Bottini *et al*., 1989). The same gibberellins were found in similar amounts in cultures of *A. brasilense* (Janzen *et al*., 1992). Treatment with either white or blue light increased the amount of GA_1 and GA_3, by two- or three-fold, respectively, as compared to dark-growth conditions (Piccoli and Bottini, 1996).

A. lipoferum USA5b produces enzymatic activities that could de-conjugate GA glucosyl conjugates, glucosyl esters (GA-G) or glucosyl ethers (GA-EG). Using

GC-MS, metabolism of the conjugates was found to produce the following GAs: GA_1, GA_3, GA_5, GA_9, and GA_{20} (Piccoli *et al*., 1996; Piccoli *et al*., 1997). In the fungus *G. fujikuroi*, both GA_3 and GA_1 are derived from GA_4 in a metabolic pathway known as early-3β-hydroxylation (Rojas *et al*., 2001). GA_{20} is an immediate precursor of GA_3 and GA_1 *via* GA_5 in maize, whereas *Phaeosphaeria* metabolizes GA_9 to GA_1 either *via* GA_4 or *via* GA_{20}. Taken together, the above results strongly suggested the occurrence of two different pathways for biosynthesis of GA_3 and GA_1 in *A. lipoferum* spp. Further, the data support the concept that the growth promotion in plants induced by *Azospirillum* infection may occur by a combination of both gibberellin production and gibberellin glucoside or glucosyl ester de-conjugation by the bacterium (Piccoli *et al*., 1997).

Tests of both *Rhizobium phaseoli* wild-type strain and derived Nod^- and Fix^- mutants for their ability to produce GAs showed that the major gibberellins excreted were GA_1 and GA_4 with smaller amounts of GA_9 and GA_{20}. The GAs pools in roots and nodules were of similar size, indicating that *Rhizobium* does not make a major contribution to the GAs content of infected tissues (Atzorn *et al.,* 1988).

6.2. Cytokinins

Little information is available as yet on the production of cytokinins by soil bacteria. Some *Azospirillum* strains are capable of producing compounds with cytokinin-like activity (Tien *et al.,* 1979), but zeatin-zeatin riboside (Z-ZR) synthesis was very limited as compared to the IAA production. Further, in contrast to the sharp rise of IAA during the stationary phase, Z-ZR production increased earlier and more slowly (Omay *et al.,* 1993).

In an ecological survey, the isolation and quantification of cytokinins was performed for a variety of bacterial strains isolated from a common grass, *Festuca*. This work included both plant pathogens, such as *A. tumefaciens*, *P. syringae* pv. *savastanoi*, and *E. herbicola* pv. *Gypsophilae*, as well as non-pathogenic bacteria, such as *Azotobacter chroococcum*, *Azotobacter beijerinckii*, *Pseudomonas fluorescens* and *P. putida*. *A. chroococcum* was the most important cytokinin producer. Physiological analysis showed that adenine and isopentyl alcohol enhanced cytokinin bioactivity. Moreover, pH 6.5, 32°C, and shaken and aerated conditions were found to be optimum for production of cytokinins derivatives, such as zeatin (Z), zeatin-riboside (Z-R), H_2Ado-dihydrozeatin (Arshad and Frankenberger, 1991). More recently, studies on cytokinin production in *P. fluorescens* led to detection of isopentyladenosine ([9R]iP), Z-R, and dihydrozeatin riboside (DHZ-R). The production was enhanced by 67% after addition of adenine to the growth medium (García de Salamone *et al.,* 2001). Characterization by HPLC, MS, radioinmunoassay and bioassay confirmed that the cytokinins, Z, Z-R, ([9R]iP), isopentenyladenine (iP), dihydrozeatin (diH)Z, and DHZ-R, were present in culture filtrates from *P. syringae* pv. *savastanoi*. When assayed during the exponential-phase of growth, the cultures produced 1000-times more cytokinin than comparable cultures of *A. tumefacciens* (Roberto and Kosuge, 1987).

6.3. Microbial Production of Ethylene and Prevention of Ethylene Synthesis

Ethylene production by bacteria, including *E. coli*, *Rhizobium trifolii*, plant pathogenic bacteria, such as *P. syringae,* and fungi, was reported a long time ago (Arshad and Frankenberger, 1991; Fukuda *et al*., 1993). Two routes for ethylene synthesis that differ from the plant pathway were described. In the route described in *E. coli*, a methionine aminotransferase converts methionine into 2-oxo-4-methylthiobutyric acid, which is in turn oxidized most probably to ethylene, methanediol, and CO_2 by a soluble NADH:Fe(III) oxidoreductase (Ince and Knowles, 1986).

A second route was found that involved a 2-oxo-glutarate-dependent dioxygenase, called EFE, as found for the plant enzyme. This activity, which catalyzes the oxidation of 2-oxo-glutarate and requires arginine, was purified from *P. syringae* pv. *phaseolicola* (Fukuda *et al*., 1993). The responsible structural gene, *efe*, which encodes the *P. syringae* enzyme, has been localized on cryptic endogenous plasmids in several strains (Nagahama *et al*. 1994). The translation product EFE shared little identity with the ACC oxidase enzymes from plants. However, because several clusters of invariant residues and hydropathy profiles were conserved, it was proposed that plant and *P. syringae* EFE might derive from a common ancestor (Fukuda *et al.*, 1993).

There is no report in the literature on the influence of microbial ethylene production on plant growth. Recently, transgenic tobacco plants containing the *P. syringae efe* gene were constructed and an increase in ethylene production was correlated with the dwarf phenotype obtained for some plants (Araki *et al*., 2000).

There is an increasing interest in bacteria that can prevent ethylene production in plants by breaking down 1-aminocyclopropane-1-carboxylic acid (AAC), the immediate precursor of ethylene. ACC deaminase activity is common in soil bacteria, yeast, and fungi (Penrose and Glick, 2003). This enzyme, which catalyses the conversion of ACC to α-ketobutyrate plus ammonia, is a homotrimeric protein that requires pyridoxal phosphate as a cofactor (Sheehy *et al*., 1991). It allows bacteria to grow with ACC as the nitrogen source. The structural gene, *acdS*, has been cloned from several *Rhizobium*, *Pseudomonas*, and *E. cloacae* strains (Sheehy *et al*., 1991; Campbell and Thomson, 1996; Shah *et al*., 1998; Belimov *et al.*, 2001). Genome projects reveal that putative ACC deaminases are also encoded by the plant pathogens, *A. tumefaciens, P. syringae* pv. *tomato, Ralstonia solanacearum*, and different rhizobia.

6.4. Other Plant Growth-Affecting Substances

Occurrence of abscisic acid at low concentrations in supernatants of *Azospirillum* cultures was reported but not further documented (Kolb and Martin, 1985; Iosipenko and Ignatov, 1995).

Another compound produced by *Azospirillum* was found to mimic the effect of IAA in several plant tests. This compound, produced when the bacteria were grown on nitrate, was identified as nitrite that was generated by the dissimilatory nitrate reductase. Because the effect of nitrite could be enhanced by ascorbate, it was

suggested that nitrite interacted with ascorbate in the plant cells and that the reaction product was responsible for the observed auxin-like response in bioassays (Zimmer and Bothe, 1988; Zimmer *et al*., 1988a).

Synthetic auxins are commonly used in agriculture. The property of degrading 2,4-D, a common herbicide, was found initially in strains of *Ralstonia* (formerly *Alcaligenes*), *R. eutropha* and *R. paradoxus*, that contained a conjugative catabolic plasmid (Don and Pemberton, 1981). The initial steps of 2,4-D mineralization involve first a α-ketoglutarate-dependent 2,4-D dioxygenase (encoded by *tfdA*) that converts 2,4-D into 2,4-dichlorophenol, and then a phenol hydroxylase (encoded by *tfdB*) that converts 2,4-dichlorophenol to dichlorocatechol. A number of different species belonging to both the α– and β–subgroups of the Proteobacteria have the ability to degrade 2,4-D. PCR amplification of *tfdA* and other *tfd* gene sequences revealed that extensive interspecies transfer has been involved in the evolution of the 2,4-D-degradation ability by these bacteria (Fulthrope *et al.* 1995).

7. PLANT GROWTH PROMOTION (PGP): ROLE OF BACTERIAL PHYTOHORMONE PRODUCTION, ACC-DEAMINASE, AND USE OF SYNTHETIC AUXINS

7.1. PGP Effect on Crops of Agronomic Importance

After inoculation with *Azospirillum*, several PGP effects are observed. They include both modifications of the root morphology, such as a dramatic increase of length and density of roots hairs, and an increase in root branching and root surface area, which lead to an enhanced uptake of water and minerals (see Chapter 7 of this volume). All these effects have been tentatively attributed to the production phytohormones, such as IAA, gibberellin, and kinetin by the bacteria (Tien *et al.,* 1979; Jain and Patriquin, 1984; 1985; Fallik *et al.,* 1988).

7.1.1. Use of Low IAA Producers

The effect of *Azospirillum* inoculation on the plant is concentration dependent, leading to either the promotion or inhibition of root growth, (Barbieri and Galli, 1993; Kapulnik *et al*., 1985; Dobbelaere *et al*., 1999). Thus, inoculation with *Azospirillum* mimics the typical growth response induced by auxins, which are inhibitory to plant growth at high concentrations and stimulatory at lower levels.

When inoculated on wheat seedlings, a mutant of *A. brasilense* (SpM7918), which produced very low quantities of IAA, was shown to be less effective in promoting the development of the root system, both in terms of number and length of lateral roots and distribution of roots hairs (Barbieri and Galli, 1993). Dobbelaere *et al.* (1999) implemented a plate-assay protocol to perform seedling-inoculation experiments with the wild-type *A. brasilense* strains, Sp245 and Sp7, and with *ipdC* mutants. They observed that inoculation with increasing cellular concentrations of the wild-type strains led to a strong decrease in root length but an increase in root-hair density, an effect similar to that produced by IAA at a concentration of 10^{-8}M. No inhibition of root length was observed when root tips

were inoculated with the *ipdC* mutants unless a high inoculum concentration (up to 10^9 cfu/ml) was used. In addition, only slightly more root hairs than on a non-inoculated control were observed (Dobbelaere *et al.,* 1999). The wild-type phenotype could be restored by addition of 0.1mM Trp (Dobbelaere *et al.,* 1999). This last result suggested that Trp can be converted into IAA, but whether this resulted from metabolism by the bacteria, *e.g.*, *via* the TAM pathway (Carreño-Lopez *et al*., 2000) or by the host plant remained unclear. It also suggested that the strong inhibitory effect observed on root length at high-cell density did not involve *ipdC*.

Work performed with the non-pathogenic *E. herbicola* 299R strain showed that *ipdC* transcription increased 32-fold *in planta* on leaves of bean and tobacco and 1000-fold on pear flowers (Brandl and Lindow, 1997). Other studies with both wild-type and *ipdC* mutants demonstrated that IAA production contributed to the epiphytic fitness of the bacteria on the bean plants and pear blossoms, because the *ipdC* mutants exhibited a ten-fold reduced fitness when compared to the wild-type strain (Brandl and Lindow, 1998).

7.1.2. Effect on the Metabolism of Endogenous Phytohormones

In corn seedling, roots inoculated with *Azospirillum* exhibited relatively higher amounts of free forms (in contrast to conjugated forms) of IAA, IBA, and gibberellin GA_3 as compared to non-inoculated controls (Fallik *et al.,* 1989; Fulchieri *et al.,* 1993; Lucangeli and Bottini, 1996). Therefore, it appears that the presence of *Azospirillum* may affect the metabolism of endogenous phytohormones in the plant. It is worth noting that the reversion of the dwarf phenotype in the *dwarf-1* line of maize and *dwarf-x* of rice mutants occurred when *A. brasilense* Cd and *A. lipoferum* op3 were inoculated onto these mutants (Lucangeli and Bottini, 1996). Moreover, when two types of GA_{20}–glucosyl conjugates, GA_{20}-G and GA_{20}-EG, were added, both were effective in promoting growth of seedlings and reversing dwarfism (Cassán *et al*., 2001). These results lead to the conclusion that both GAs production and hydrolysis of GA-conjugates by *Azospirillum* species could be an important mechanism that accounts for the beneficial effect observed when plants are inoculated with these bacteria. In addition, after application of uniconazole (an inhibitor of GA synthesis) to maize, GA_3 could not be detected in non-inoculated plants in contrast to plants inoculated with *Azospirillum* (Lucangeli and Bottini, 1997).

7.1.3. Sugarcane Promotion

Up to 80% of the total fixed-N incorporated into several sugarcane cultivars can be attributed to BNF (see Chapter 11 of this volume). In addition, the promotion of sugarcane growth could be driven by a hormone-dependent mechanism. Under fixed-N-sufficient growth conditions, plants inoculated with *Gluconacetobacter diazotrophicus*, either as the wild type or a *nifD* mutant, are *ca*. 20% taller than non-inoculated plants. These results suggested that *G. diazotrophicus* could benefit sugarcane in two ways: (i) by transfer of bacterially fixed-N; and (ii) *via* phytohormone production (Sevilla *et al.,* 2001). Inoculation of sorghum seedlings

with *G. diazotrophicus* increased overall growth, but it had a moderate effect on the increase of total carbohydrates, such as glucose and fructose (Bastían *et al*., 1999).

7.2. Gain in Root Length Associated with ACC Deaminase

Ethylene has an inhibitory role on root elongation. A role for ACC deaminase in preventing the ethylene effect was shown in inoculation experiments of canola roots with *E. cloacae*. Using a mutant strain with an interrupted *acdS* gene, Li *et al*. (2000) showed that the ability to promote root elongation was diminished as compared to that induced by the wild type. Similar results were observed with a *P. putida* strain (see details in Chapter 7, section 2.4, of this volume). The authors proposed that a major mechanism that accounted for the plant growth-promotion effect is linked to the lowering of plant ethylene levels by the bacterial ACC deaminase. Therefore, this research group introduced the *acdS* gene into *Azospirillum*, which does not normally display ACC-deaminase activity. The resulting strains displayed high ACC-deaminase activity, which correlated with an increased ability to stimulate root growth of both tomato and canola, but not of wheat seedlings (Holguin and Glick, 2001).

*7.3. Root Deformation (*para*-Nodules) Induced with Synthetic Auxins*

Morphological changes, often referred to as "pseudo-nodules" and linked to the application of Trp, IAA, or synthetic auxins (2,4-D, NAA, and others), were first recorded more than 60 years ago. However, Y.F. Nie, in China, first reported that addition of 2,4-D to rice roots induced deformations that can be colonized by nitrogen-fixing bacteria (reviewed in Tchan and Kennedy, 1989; Cocking *et al.,* 1994). The synthetic auxin-induced deformations could be obtained with several non-leguminous plants, including rice, wheat, barley, and oil-seed rape. Although these structures differ from nodules and are merely modified lateral roots (Rolfe *et al.*, 1997), they were commonly called nodule-like structures, pseudo-nodules or *para*-nodules (Kennedy and Tchan 1992; Kennedy *et al*., 1997). Nitrogenase activity was observed *in situ* when the 2,4-D-treated plantlets inoculated with *Azospirillum* were placed at reduced O_2 tension (Zeman *et al*., 1992). These structures could be colonized by several bacterial species, including *Azospirilla, Derxia, Gluconacetobacter, Herbaspirillum,* and rhizobia (Kennedy *et al*., 1997; Rolfe *et al*., 1997).

Azospirillum is an efficient root colonizer (see Chapter 5 of this volume). In association with plants, the bacteria differentiate into non-flagellated cyst-like forms (Katupitiya *et al.,* 1995; Pereg-Gerk *et al*., 1998). Inoculation of 2,4-D-treated wheat seedlings with *Azospirillum* strains allowed the bacteria to colonize the induced root deformations. The bacteria were found intercellularly, usually in the basal zone of the "*para*-nodules" where the wheat-plant cells appear loosely packed (Katupitiya *et al.,* 1995). Ammonia-excreting *Azospirillum* mutant strains were localized both inter- and intra-cellularly in the 2,4-D-induced root deformations in maize (Christiansen-Weniger and Vanderleyden, 1994). The mutant strain, Sp7-S,

which is impaired in capsule formation and remains in the vegetative form when associated with roots, colonized these structures more efficiently than the wild type, although the colonization of the root surface of control plants (not treated with 2,4-D) was less efficient as compared to the wild type (Katupitiya *et al.,* 1995; Pereg-Gerk *et al*., 1998). Using a transcriptional *nifH-lacZ* fusion, higher β-galactosidase activity was observed with this mutant than with the wild type in 2,4-D-treated plants, consistent with the endophytic mode of colonization of the Sp7-S mutant strain (Katupitiya *et al.,* 1995; Kennedy *et al.,* 1997; Pereg-Gerk *et al*. 2000).

8. CONCLUDING REMARKS

A full understanding of IAA, gibberellin, and cytokinin metabolism calls for additional identification and analysis of the intermediates, enzymes, and genes involved in their biosynthesis, as well as in the isolation of mutants defective in each pathway. Although the production of phytohormones at the free-living state is well established in many microorganisms, there is still insufficient evidence for their synthesis in their natural habitats. The ecological significance of phytohormone production by bacteria would be more convincing if bacterial production of phytohormones could be demonstrated while the bacteria were colonizing the root system. Because the plant and the bacteria both synthesize and secrete auxins, gibberellins, and cytokinins it is difficult to address the contribution of one particular hormone as responsible of the effects observed. Thus, the possibility that the host plant directs the bacterium to produce IAA using the Trp present in root exudates is intriguing but remains speculative at this point.

The enzymes and intermediates in IAA biosynthesis have not yet been definitively established, although substantial progress has been made on the biochemical characterization of these pathways. Much of the evidence for the importance of IAA production in plant-microbe beneficial interactions comes from the use of attenuated mutants and their relationship to the concomitant attenuation of the characteristic biological effects. In the future, the use of transcriptional (or other types) fusions for the analysis of the differential expression of the bacterial genes involved in the pathways of phytohormone biosynthesis, when associated with the host plant, should generate important information.

In recent years, a number of studies in Belgium, Israel, France, Argentina, Uruguay, México, USA, and South Africa on the inoculation of cereals, such as wheat, maize, sugarcane, sorghum, and sunflower, with PGPR have reported beneficial effects, such as an increase in nitrogen content and in yield. The success of field experiments depends of many parameters, such as the strain used, the concentration of bacterial inoculum, the viability of bacteria during storage, the carrier employed, appropriate inoculation methodology, and soil characteristics. The identification of many traits and genes related to the beneficial effects of the inoculated bacteria should result in a better understanding of the performance of bioinoculants in the field. It will also provide a strategy to design genetically modified strains with improved PGP effects.

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Chapter 7

THE PLANT GROWTH-PROMOTING EFFECT AND PLANT RESPONSES

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1. N_2 FIXATION VS. "HORMONAL" EFFECTS: HISTORICAL PERSPECTIVES.

Following the report of the isolation, properties, and biological nitrogen fixation of the then-called *Spirillum lipoferum* (later renamed *Azospirillum*) when associated with tropical grasses and maize (Döbereiner and Day, 1976), there was a world-wide effort to demonstrate substantial biological nitrogen fixation by *Azospirillum* and the significance of its contribution to the fixed-nitrogen need of the plants. Inoculation experiments were carried out under controlled and field conditions, using different plant-growth systems and different plant species. Biological nitrogen fixation (BNF) was measured by the acetylene-reduction assay (ARA), ^{15}N-dilution technique, $^{15}N_2$ fixation, and Kjeldahl N-content measurements (see Okon, 1985). Although in inoculated plants as compared to non-inoculated controls, it was possible to measure many-times higher ethylene-production rates by ARA (see, *e.g.*, Kapulnik *et al.,* 1987), the values when extrapolated to fixed-nitrogen did generally not amount to more than 10 kg N ha^{-1} $year^{-1}$. Nevertheless, reports of both high ethylene-production rates by ARA assays in detached roots after 24h of incubation and by ^{15}N-dilution measurements in some cultivars of sugarcane indicated a potential for significant BNF (Döbereiner, 1992). This potential maintained the research interest in associative symbioses.

At the same time, in inoculation experiments under both controlled plant-growth conditions and in the field, beneficial effects on plant growth and yield of many agronomically important crops were repeatedly observed, together with remarkable

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changes in the morphology of inoculated root systems (Tien *et al.,* 1979; Pacovsky, 1990; Sarig *et al*., 1992; Dobbelaere *et al.*, 2001). It became clear that the positive effects of *Azospirillum* on plant growth are mainly derived from morphological and physiological changes of the inoculated plant roots, which lead to an enhancement of water and mineral uptake (Okon and Kapulnik, 1986; Fallik *et al.*, 1994). This conclusion is in agreement with the observation that the effects of inoculation are highest in fields moderately fertilized with N, P and K, indicating that inoculation with *Azospirillum* does not replace N-fertilizer but rather improves its utilization, leading to the same crop productivity at lower levels of fertilizer (Okon, 1985).

Because *Azospirillum* clearly and consistently promoted the growth of plants, the organism was classified as a Plant-Growth-Promoting Rhizobacterium (PGPR). In general, beneficial rhizobacteria that stimulate plant growth are referred to as PGPR (Davison, 1988; Kloepper *et al.*, 1989), a group that includes different bacterial species and strains belonging to genera such as *Acetobacter* (now *Gluconacetobacter*), *Azospirillum*, *Azotobacter*, *Bacillus, Burkholderia, Herbaspirillum,* and *Pseudomonas* (Weller and Thomashow, 1994; Glick, 1995; Probanza *et al*., 1996).

This chapter deals mainly with the responses of plants to *Azospirillum* (as a model PGPR) inoculants. Although bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium, Mesorhizobium,* and *Azorhizobium* are known for their capacity to fix N_2 in a symbiotic relationship with the roots of leguminous plants, they are usually not considered as PGPR in this highly specific symbiotic interaction. However, Rhizobiaceae also have the ability to form nonspecific associative interactions with roots of other plants without forming nodules (Reyes and Schimidt, 1979). They were found to attach to the surface of monocots in the same manner as they attach to those of dicot hosts (Shimshick and Hebert, 1979; Terouchi and Syõno, 1990). Furthermore, they are able to stimulate the growth and increase the yield of these non-legumes both in greenhouse and field experiments (Biswas *et al*., 2000; Yanni *et al*., 2001) and, therefore, they can also be considered as PGPR in these cases. This chapter is restricted to plant growth promotion by either free-living or associative N_2-fixing bacteria as well as some endophytic diazotrophs. *Rhizobium* will only be discussed when used as a PGPR with non-legumes.

2. EFFECTS OF *AZOSPIRILLUM* AND OTHER DIAZOTROPHS ON ROOT MORPHOLOGY

2.1. Root Hairs

One of the most pronounced effects of inoculation with *Azospirillum* on root morphology is the proliferation of root hairs (Figure 1). Inoculation of several cultivars of wheat, maize, tomato, sorghum, foxtail millet, pearl millet, and other grasses with several strains of *Azospirillum* caused morphological changes in the root, including an increase in the number and density of root hairs and a shortening of the time for appearance of root hairs (Tien *et al*., 1979; Umali-Garcia *et al*., 1980; Martin and Glatzle, 1982; Patriquin *et al.*, 1983; Okon, 1984; Kapulnik *et al*.,

1985b; Hadas and Okon, 1987; Morgenstern and Okon, 1987a; Barbieri *et al*., 1991). Inoculation also increased the length of mature root hairs and shortened the distance between the root apex and the region at which root hairs start to elongate (Harari *et al*., 1988; Dobbelaere *et al*., 1999). Likewise, inoculation of seeds of different plant species (canola, tomato and wheat) with cultures of *Azotobacter paspali* led to the formation of more root hairs that were longer and thicker than those of control plants (Abbass and Okon, 1993).

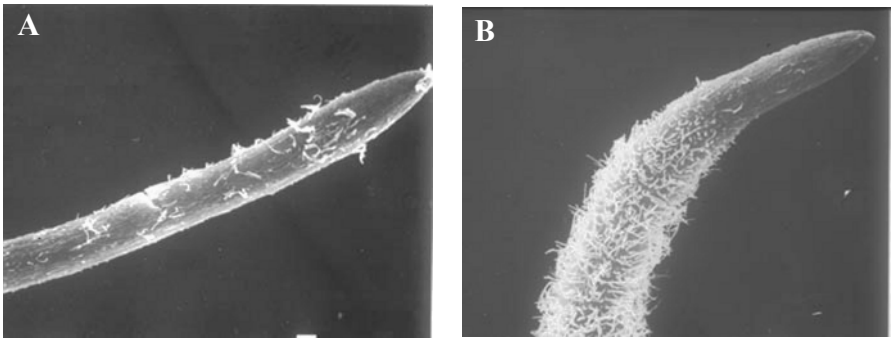

Figure 1. Scanning electron microphotographs of the effect of inoculation with A. brasilense *on the morphology of tomato root tips 48 hrs after inoculation (x36). (A) Non-inoculated control root tip. (B) Root tip inoculated with* 10^8 *cfu* ml^{-1}. *Reprinted from Hadas and Okon, 1987, with permission of Springer-Verlag.*

This effect on root-hair proliferation was strongly influenced by, and varied with, inoculum level (Kapulnik *et al.,* 1985a; Okon and Kapulnik, 1986; Hadas and Okon, 1987; Morgenstern and Okon, 1987a; Harari *et al*., 1988; Dobbelaere *et al*., 1999). Low inoculum concentrations (10^5 colony forming units (cfu) ml^{-1}) caused denser and longer root hairs, without affecting the length of the root-elongation zone (the distance from root cap to the first root hair, 50 µm in length), suggesting that the effect is due to an earlier initiation of root-hair formation. Higher bacterial concentrations ($\geq 10^8$ cfu ml^{-1}) caused an asymmetrical growth of the root tip and an expansion of the root diameter. At the same time, there was a pronounced shortening of the root-elongation zone with a much higher density of root hairs that covered all the elongation region of the root, including the meristematic region near the root cap. It is not clear whether these effects on root-hair formation are caused: (i) by inhibition of root elongation by affecting either cell division or cell elongation at the elongation zone; (ii) by increased formation of root hairs (differentiation of cells from the root elongation zone to form root hairs); or (iii) by a combination of the above factors (Okon and Kapulnik, 1986).

Similarly to what was observed in several grasses and cereals, inoculation with *Azospirillum* was also found to increase the root-hair density, root-hair length, and root diameter of several legumes, including common bean (Burdman *et al*., 1996), alfalfa (Itzigsohn *et al*., 1993), and clovers (Plazinski and Rolfe, 1985), as compared

to non-inoculated control plants or plants inoculated with *Rhizobium* alone. This promotion of root-hair formation might be the reason for the enhanced nodulation observed after combined inoculation with *Azospirillum* and *Rhizobium* (Volpin and Kapulnik, 1994; Okon and Itzigsohn, 1995; Burdman *et al*., 2000) because *Rhizobium* infection takes place by the formation of infection threads in root hairs (Long, 1989). On the other hand, many data indicate that the roots of non-legumes can also be affected by symbiotic bacteria; bradyrhizobia and rhizobia were found to induce root-hair curling on maize, rice and oat plants (Plazinski *et al*., 1985; Terouchi and Syõno, 1990).

Most of this stimulation of root growth is assumed to result from the production of phytohormones by the bacterium (see Chapter 6 of this volume) because the morphological changes of the plant root following *Azospirillum* inoculation could be mimicked by applying a combination of plant growth substances (Jain and Patriquin, 1985; Tien *et al.*, 1979). Promotion of root growth, especially the increase of hair density in zones physiologically active for nutrient uptake and water absorption, could lead to better soil exploration and also improve the growth and development of the plants (Fulchieri *et al*., 1993).

2.2. Root Hair Deformation and Branching

Apart from promoting root-hair development, inoculation with *Azospirillum* (10^9–10^{10} cfu ml^{-1}) also caused branching of root hairs. Two types of root-hair branching were distinguished in wheat; one produced branches of equal length (the tuning-fork-like deformation) and the other branches of unequal length (Patriquin *et al*., 1983). This root-hair deformation was found to be somehow strain-specific, with homologous strains, which were isolated from surface-sterilized roots of the same crop that was to be inoculated, inducing the formation of more tuning forks than non-homologous strains. No or very few tuning forks were found in non-inoculated plants (Jain and Patriquin, 1984). Jain and Patriquin (1985) identified a substance produced by *Azospirillum* that caused branching of wheat root hairs as indole-3-acetic acid (IAA) and they could mimic the branching effect by using pure solutions of IAA.

The role, if any, of root-hair branching in the colonization of roots by *Azospirillum* is not known. The phenomenon is significant, however, in that it is a predictor of the potential growth response of wheat to inoculation with *Azospirillum* (Jain and Patriquin, 1984). Strains that caused the most tuning forks in the laboratory also brought about the greatest increase in plant N in a separate field experiment (Baldani *et al*., 1983).

2.3. Cross Sections

Cross-sections of corn and wheat roots inoculated with *Azospirillum* showed an irregular arrangement of cells in the outer four or five layers of the cortex (Figure 2; Lin *et al*., 1983; Kapulnik *et al*., 1985b). Photomicrographs of cross sections, taken near the root tip shortly after inoculation of burr medic with 10^9 cfu ml^{-1} of

Azospirillum brasilense strain Cd, showed larger cortical cells. However, their number (in cross sections) did not increase as compared to controls (Yahalom *et al*., 1991).

The DNA concentration in root segments of burr medic inoculated with 10^9 cfu ml^{-1} of *Azospirillum* was significantly lower than that in roots inoculated with 10^7 cfu ml^{-1} of *Azospirillum* or in controls. From this finding, it was concluded that the reduction in root growth might be the result of a decrease in cell division in the apical meristem of the root (Yahalom *et al*., 1991).

These effects of inoculation on cell arrangement and size may be due to the production of plant-growth promoting substances: (i) by the colonizing bacteria; (ii) by the plant as a reaction to colonization; or (iii) by the action of pectic enzymes produced by the bacteria (Okon and Kapulnik, 1986).

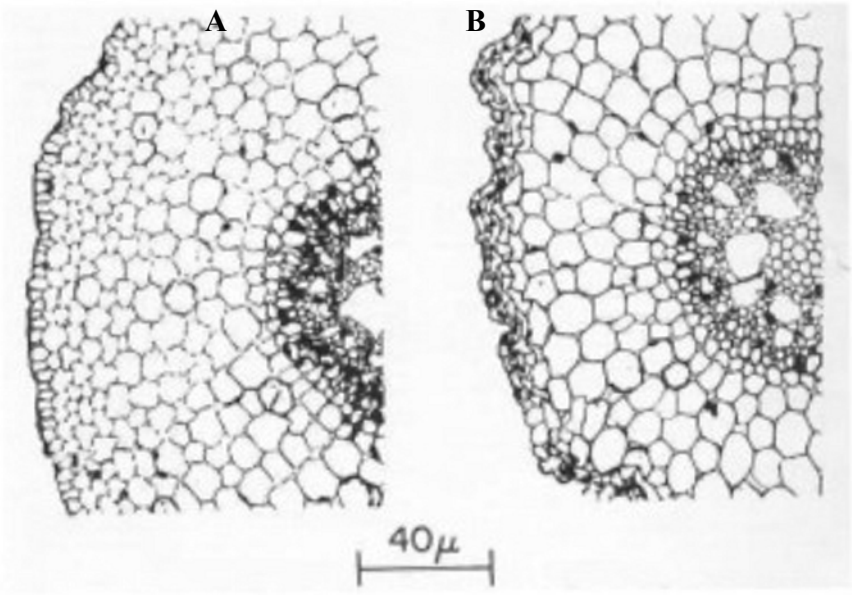

Figure 2. Light micrographs of the cross section of corn roots.
(A) Control tissue; (B) Sp 7-inoculated tissue; bar, 40 μm.
Reprinted from Lin et al.*, 1983, with permission of American Society for Microbiology.*

2.4. Root Elongation

In addition to stimulating root-hair formation, inoculation with diazotrophs can also promote the elongation of primary roots. Increases in root length were observed after inoculation of several cultivars of wheat, sorghum, proso millet, sugar beet, rice, and tomato with *A. brasilense* (Kapulnik *et al*., 1985a; Kolb and Martin, 1985;

Marschner *et al*., 1986; Hadas and Okon, 1987; Harari *et al*., 1988; Murty and Ladha, 1988; Levanony and Bashan, 1989; Sarig *et al*., 1992) and after inoculation of canola, lettuce, tomato, and wheat with *Pseudomonas putida* GR12-2 (Lifshitz *et al*., 1987; Hall *et al*., 1996). Early seedling root growth of the non-legumes, canola and lettuce, was significantly promoted by inoculation of seeds with certain strains of *Rhizobium leguminosarum* (Noel *et al*., 1996).

Here again, the effect is dependent on the bacterial concentration applied. Low concentrations (10^3–10^6 cfu ml^{-1}) stimulated root elongation, whereas high inoculum concentrations (above 10^7 cfu ml^{-1}) inhibited root growth (Kapulnik *et al*., 1985b; Harari *et al*., 1988). The concentration-dependent effect on root length is consistent with the effects of plant-root treatment with high concentrations of exogenous IAA. Depending on the plant species, exogenous IAA concentrations above a threshold of about 10^{-6}–10^{-9} M are inversely proportional to root elongation (Pilet and Saugy, 1985; Scott, 1972). By using a mutant strain of *A. brasilense* impaired in IAA production, it was indeed demonstrated that the effect of inoculation on wheat root elongation was mainly due to the production of IAA by this bacterium (Dobbelaere *et al*., 1999).

In contrast, the nitrogen-fixing strain *P. putida* GR12-2 promotes root elongation by lowering the high levels of ethylene in plants (Glick, 1995; Glick *et al*., 1994; 1998). Low levels of ethylene appear both to enhance root initiation and growth and promote root extension, whereas higher levels of ethylene can lead to inhibition of root elongation (Mattoo and Suttle, 1991; Ma *et al*., 1998). *P. putida* GR12-2 possesses the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate biosynthetic precursor of ethylene in plants (Honma and Shimomura, 1978; Yang and Hoffman, 1984; Walsh *et al*., 1981). Rather than stimulating root elongation *per se*, the bacterium actually prevents the inhibition of root elongation by high levels of ethylene.

2.5. Root Branching/Lateral Root Formation/Adventitious Root Formation

Inoculation of wheat seedlings with *A. brasilense* increased the number and length of the lateral roots (Barbieri *et al*., 1986; 1988). Similar results were obtained with pearl millet (Tien *et al*., 1979), with hybrid *Sorghum bicolor x Sorghum sudanense* (Morgenstern and Okon, 1987a), and with sugar beet (Kolb and Martin, 1985; Marschner *et al*., 1986).

Also, combined inoculations of diazotrophs with *Rhizobium* influenced the number of lateral roots. Yahalom *et al*. (1991) found that inoculation of burr medic seedlings grown in pouches with *A. brasilense* strain Cd at a concentration of 10^6 cfu ml^{-1} significantly increased the number of lateral roots. Petersen *et al*. (1996) reported increased lateral-root formation after co-inoculation of common bean seedlings with *Bacillus polymyxa* and *Rhizobium etli*.

In a field experiment with wheat, inoculation with *Azospirillum* was found to increase the total number of roots per plant as well as the number of roots per tiller (Figure 3; Kapulnik *et al*., 1987). In a maize field experiment with *A. brasilense* strain Cd carried out in Israel in 1998, it was observed that the number of adventitious roots and the total adventitious-root length increased significantly (P =

0.05) above non-inoculated controls by 24% and 41%, respectively, 2 weeks after emergence (Table 1; Dobbelaere *et al*., 2001).

These morphological modifications of the root system could also improve the mineral uptake from the environment (Okon, 1985).

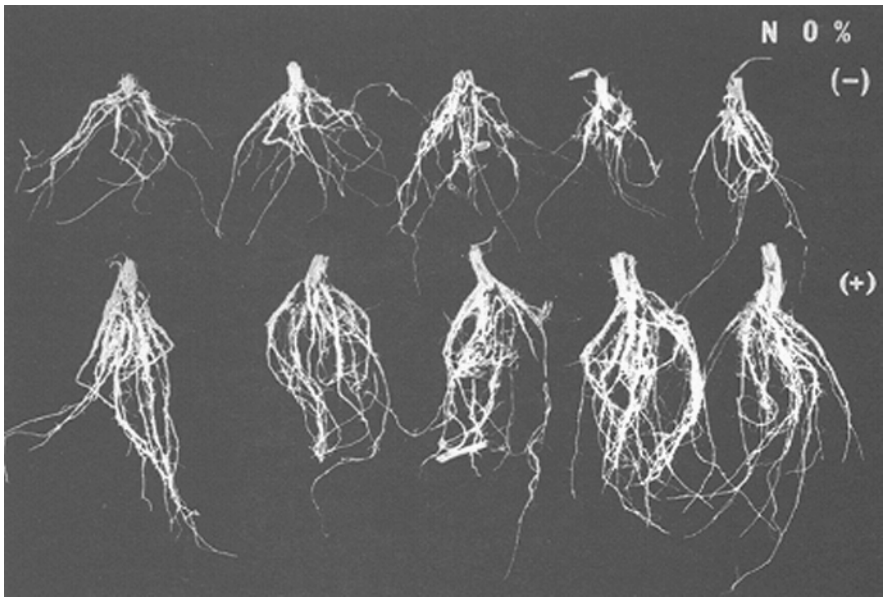

Figure 3. Effect of Azospirillum *inoculation on root development of wheat cultivar Miriam at flowering under field conditions in unfertilized soil.*
(-) non-inoculated control; (+) inoculated.
Reprinted from Kapulnik et al.*, 1987, with permission of Springer-Verlag*

Table 1. Effect of Azospirillum *on the formation of adventitious roots by field-grown maize 14 days after sowing.*
Values in the same column followed by a different letter are significantly different at P = 0.05.

	Number	Length (cm)[1]	Total length (cm)
Azospirillum	4.2 a	15.4 a	60.9 a
Control	3.4 b	12.3 a	43.2 b

[1] *Average of individual adventitious roots*

2.6. Early Nodulation (Legumes)

Structures that have been tentatively called either "nodule-like structures" or hypertrophies formed by rhizobia have not only be found on legumes, but have also been observed on oilseed rape, mustard, Arabidopsis, rice, and other non-legumes (Al-Mallah *et al*., 1990; Ridge *et al*., 1992; Trinick and Hadobas, 1995). Some isolates of *Bradyrhizobium* sp. can form nitrogen-fixing nodules with *Parasponia*, a non-legume belonging to the family *Ulmaceae* (Trinick, 1973; Werner, 1992).

Several non-symbiotic diazotrophs are known to promote nodulation of legumes when used in combination with rhizobia. Co-inoculation of alfalfa, burr medic, vetch, garden and chickpea (Figure 4), white clover, common bean, winged bean, and soybean with *A. brasilense* or *A. lipoferum* (Singh and Subba Rao, 1979; Iruthayathas *et al*., 1983; Plazinski *et al*., 1984; Sarig *et al*., 1986; Yahalom *et al*., 1987; 1990; Itzigsohn *et al*., 1993; Burdman *et al*., 1997) resulted in earlier nodulation and increases in total nodule number as compared to plants inoculated with their respective rhizobial symbiont alone. Similar results were obtained with soybean, cowpea, and clover additionally inoculated with *Azotobacter vinelandii* (Burns *et al*., 1981) and with common bean additionally inoculated with *B. polymyxa* (Petersen *et al*., 1996).

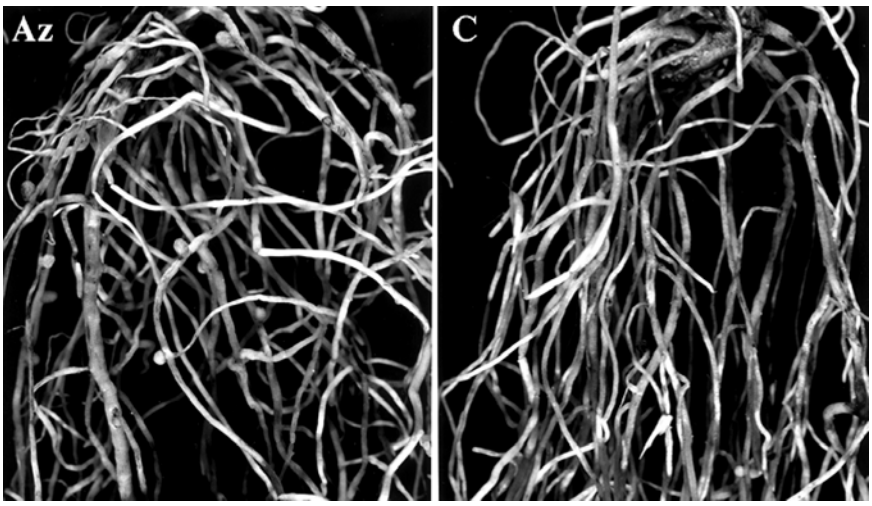

Figure 4. Effect of A. brasilense *strain Cd on nodulation of chickpeas as observed in a greenhouse experiment 84 days after sowing. Az: root inoculated with* Azospirillum*; C: non-inoculated control root. Reprinted from Hamaoui* et al.*, 2001, with permission of EDP Sciences.*

Dual inoculation with *Rhizobium* and *Azospirillum* significantly increased both upper and total nodule number of several legumes as compared to inoculation with

Rhizobium alone (Table 2; Burdman *et al*., 1997). The increase in the number of upper nodules (those present in the upper 2 cm of the roots) could indicate earlier nodulation (because upper nodules form earlier than others) or a greater susceptibility of roots to nodulation or both (Okon *et al*., 1995; Burdman *et al*., 2000). Furthermore, Iruthayathas *et al*. (1983) found that co-inoculation also influenced the distribution of nodules within the root zone. Combined inoculation produced nodules mostly on the lower part of the lateral-root branches, whereas *Rhizobium* alone mostly produced crown nodules (clustered at the base of the plant).

Still little is known about the mechanisms involved in enhanced nodulation after dual inoculation. Stimulation of nodulation may occur as a result of a direct response of the plant to *Azospirillum* inoculation, *e.g*., an increase in number of root hairs, root-hair branching, and lateral roots (Okon and Kapulnik, 1986), but also by the differentiation of a greater number of epidermal cells into root hairs that can be infected (Yahalom *et al*., 1990). *Rhizobium* infection takes place by the formation of infection threads in root hairs and, by stimulating epidermal cells to differentiate into "infectable" root hair cells, *Azospirillum* may increase the root's potential for nodule initiation (Yahalom *et al*., 1987).

Table 2. Nodule number of common bean plants inoculated with Rhizobium *at 10^5 cfu ml^{-1} either alone or in co-inoculation with* Azospirillum *at $5x10^6$ cfu ml^{-1} under gnotobiotic conditions. Plants were harvested 5 weeks after sowing. Values indicate means of 7 replicates per treatment. Different letters indicate significant differences at P= 0.05 (Burdman et al., 1997).*

	Number of nodules	
	Upper[a]	Total
Rhizobium	144 b	424 b
Rhizobium + *Azospirillum*	228 a	556 a

[a] *Nodules formed in the upper 2 cm of the root system*

Burns *et al*. (1981) suggested that enhanced nodulation by *Azotobacter* was mainly through influencing nodule initiation and not either nodule growth or function. Iruthayathas *et al*. (1983), on the other hand, found that the main-root nodule count increased with combined inoculation with *Azospirillum*, despite the fact that the same number of infection threads was formed. This may imply that there was no positive effect of combined inoculation on the regulation of nodule initiation at the stage of infection-thread formation and that the increased nodule number must be regulated at a later stage.

Furthermore, it was suggested that changes in root-hair differentiation were caused by plant growth-promoting substances (auxins, cytokinins, gibberellins) produced by *Azospirillum* (Plazinski and Rolfe, 1985; Yahalom *et al*., 1990). Both the positive and the negative effects of *Azospirillum* on nodulation and root development could be mimicked, in some cases, by application of phytohormones,

such as auxins and cytokinins (Itzigsohn *et al*., 1993; Plazinski and Rolfe, 1985; Yahalom *et al*., 1990). Apart from their direct effect on root morphology, phytohormones may also influence the nodulation process itself (Syono *et al*., 1976; Yahalom *et al*., 1987). In the case of *Azotobacter*, a non-specified protein produced by the bacterium was suggested as a possible mechanism for nodulation enhancement (Burns *et al*., 1981).

3. EFFECTS ON ROOT FUNCTION

3.1. Respiration and Respiratory Enzymes

In a Petri-dish system, inoculation of tomato with 10^8 cfu ml^{-1} of *A. brasilense* strain Cd increased the total respiration rate per root by 70% over non-inoculated controls (Hadas and Okon, 1987). Respiration rates of roots of sorghum (Sarig *et al*., 1992), maize, and common bean (Vedder-Weiss *et al*., 1999) were also increased by inoculation with *Azospirillum*. An increased root-respiration rate indicates an increase in metabolic activity. The specific respiration rate, expressed as micromoles of O_2 per minute and per milligram of root dry weight, was significantly lower in inoculated roots, suggesting that less energy was spent per gram of dry material that was formed.

Application of *A. brasilense* (10^7 cfu $plant^{-1}$) to maize plants led to an increase in the specific activity of several enzymes in root extracts; these included alcohol dehydrogenase, glutamine synthetase, isocitrate dehydrogenase, malate dehydrogenase, pyruvate kinase, and shikimate dehydrogenase. A specific-activity increase of these enzymes was observed in inoculated roots between the 2nd and 3rd week after sowing as compared to non-inoculated controls (Fallik *et al*., 1988).

3.2. Phosphatase

Acid phosphatase is involved in the breakdown of organic phosphate compounds. The specific activity of acid phosphatase in extracts of inoculated maize roots is significantly increased at the 2nd, 3rd and 4th week after sowing as compared to that in non-inoculated controls (Fallik *et al*., 1988). Thus, the increased phosphate uptake observed in roots inoculated with *Azospirillum* could result from an increase in acid phosphatase activity (see section *3.5*).

3.3. Phenyl Propanoid Metabolism and Production of Flavonoids (Legumes)

Inoculation of maize and alfalfa with *Azospirillum* did not affect the activity of phenylalanine-ammonia-lyase (PAL) and glucose-6-phosphate dehydrogenase, enzymes that are generally elevated in plants as a result of bacterial, fungal or viral infection (Fallik *et al*., 1988). Enzyme activities and mRNA levels of the defence related proteins, PAL, chalcone isomerase (CHI), and isoflavone reductase (IFR), were not induced in alfalfa by inoculation with *Azospirillum* (Volpin *et al*., 1996). These findings suggest that *Azospirillum* does not behave as a pathogenic rhizosphere bacterium.

Inoculation of either common bean or alfalfa with *Azospirillum* resulted in the production of plant-root exudates with an increased capacity to induce *Rhizobium nod*-gene expression as compared to exudates of the non-inoculated controls (Figure 5). This correlated with a change in the chemical composition of the root exudates of inoculated plants. Indeed, both the quantity and the quality of flavonoids, which are known to induce the *nod*-genes, were modified (Burdman *et al*., 1996; Volpin *et al*., 1996).

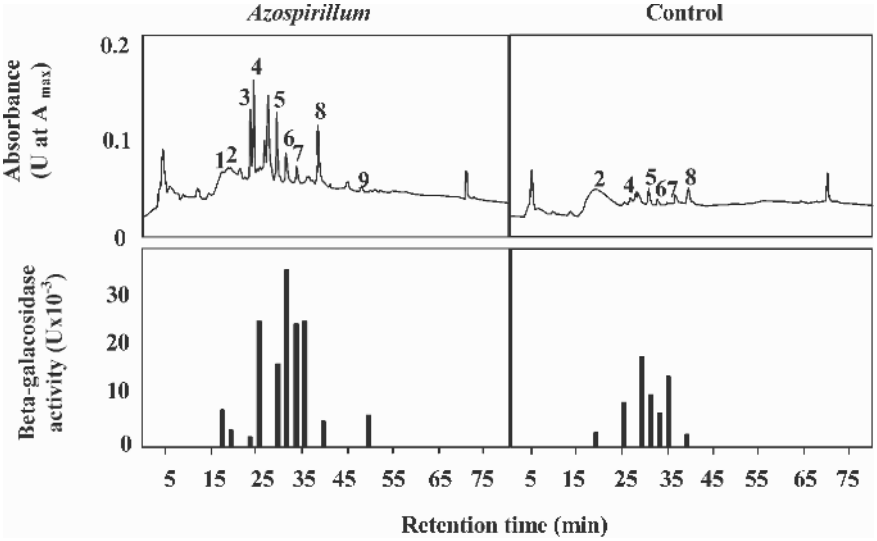

Figure 5. HPLC characteristics ($A_{240\text{-}400}$) *(top panels) and* nod*-gene induction assays (bottom panels) of fractions from root exudates of 6-day-old bean seedlings inoculated or not inoculated with* A. brasilense *at* 10^7 *cfu* ml^{-1}.
Root exudates were collected at 48 hrs after inoculation.
Peak 5 = daidzein; Peak 6 = naringenin; Peak 7 = genistein; Peak 8 = coumestrol.
Reprinted from Burdman et al.*, 1996, with permission of American Society for Microbiology.*

The presence of *Azospirillum* in the rhizosphere was reported to elicit or activate the hydrolysis of conjugated phytohormones and flavonoids in the root tissue, thus, bringing about the release of the compounds in their active forms (Fallik *et al*., 1989, 1994; Fulchieri *et al*., 1993; Burdman *et al*., 1996; Volpin *et al*., 1996). These observations suggest that there might be an increase in hydrolysis of conjugates mediated by β-glucosidases. β-glucosidases catalyze the hydrolysis of glycosidic linkages in both aryl and alkyl β-glucosidases and cellobiose and occur in all living organisms. Almost all known β-glucosidases have a subunit molecular weight of 55-65 kDa, an acidic pH for optimal activity (pH 5-6), and an absolute requirement for a β-glucoside substrate.

Plant β-glucosidases participate in defence mechanisms against pathogens by releasing compounds, such as thiocyanates, coumarins, hydroxamic acid, and terpenes. In addition, they catalyze the hydrolysis of non-active conjugates, such as glycosides of flavonoids (Hartwig and Phillips, 1991) and phytohormones (auxins, gibberellins, cytokinins and abscisic acid; see section *3.4*), thus, releasing their biologically active aglycones (Campos *et al*., 1993). At the same time, β-glucosidase activity could affect the release of sugar radicals that might be involved in the higher respiration rates that are observed in inoculated roots of maize and common bean when compared to non-inoculated controls (Vedder-Weiss *et al*., 1999).

It has been consistently observed, in both root tips and detached roots of maize and common bean, that treatment with *Azospirillum* reduced the K_m and V_{max} values of β-glucosidase activity in crude extracts. *In vitro* inoculation of detached root segments caused a decrease in β-glucosidase kinetic values. In this case, changes in K_m and V_{max} were observed as soon as 5h and the values further decreased up to 24h. This effect was shown to directly correlate with the concentration of the inoculum. Reduction in the V_{max} and K_m values may suggest a lower total activity, but a higher affinity for the substrate of specific β-glucosidases (Vedder-Weiss *et al*., 1999).

3.4. Auxin, Gibberellin, and Ethylene in Plant Metabolism

Because many diazotrophs as well as plants can synthesize phytohormones (see Chapter 6 of this volume), the effect of diazotrophic bacteria on endogenous plant-hormone levels, irrespective of the plant's own hormone production, has to be taken into account (Fallik *et al*., 1989).

Inoculation of maize seedlings growing in nutrient solution with *Azotobacter chroococcum* resulted in a synergistic increase in phytohormone concentrations (IAA, abscisic acid, isopentenyladenosine, zeatinriboside, and dihydrozeatin riboside) in comparison to the sum of hormone production by both sterile plant roots and bacteria cultures (Müller *et al.*, 1989). However, it is hard to distinguish between the hormones synthesized by the plant in response to PGPR stimulation and the hormones synthesized by the PGPR itself (Fallik *et al*., 1989). Therefore, all possibilities, the plant synthesizing more phytohormones in the presence of the bacterium, the bacteria producing and exporting more phytohormones under the influence of plant roots, or both, remain open.

A. brasilense was found to affect the amount of both free and bound IAA in the inoculated roots of maize seedlings (Fallik *et al*., 1989). Normally, about 95-98% of the IAA in maize seed is found as an ester conjugate of IAA (Epstein *et al*., 1980), which is then metabolized during seed germination to give active free IAA (Nowacki and Bandurski, 1980; Nonhebel *et al*., 1985). Inoculation of maize seedlings with *Azospirillum* resulted in a simultaneous decrease in the amount of conjugated IAA and a sharp, significant increase in the free IAA concentration compared to non-inoculated control roots. Moreover, after feeding IAA to inoculated and non-inoculated seedlings, IAA and IBA were identified only in the roots of the inoculated seedlings. When IAA is supplied to non-inoculated plant roots, it is usually conjugated rapidly (Bandurski, 1980). The presence of free IAA in the inoculated roots, which were fed with IAA for 24h, and its absence from the

non-inoculated control may be attributed either to hydrolysis of the conjugated IAA by the bacteria to release free IAA or to blocking of the conjugation of the exogenous IAA by the bacteria.

Inoculation with *A. lipoferum* had substantial effects on the gibberellin (GA) content of the roots of corn seedlings (Fulchieri *et al*., 1993). GA_3 was found as the free acid in extracts of roots of seedlings inoculated with *A. lipoferum*, whereas in extracts of roots of non-inoculated plants, it was only found after hydrolysis of the glucosyl-conjugate fraction.

In all these cases, it is not clear if the relatively higher amounts of free IAA and GA_3 in the root tissue of inoculated maize were derived: (i) from plant growth promoting substances (PGS) excreted by the colonizing bacteria; (ii) by changes in plant-hormone metabolism caused by excretion of PGS by the bacteria; (iii) by higher respiration rates of the roots demanding more glycosidic residues from hydrolysed hormonal conjugates, thus freeing IAA and GA_3; or (iv) by enzymes liberated by the bacteria that are responsible for de-conjugation of glucosylated forms. Hydrolysis of gibberellin-glucosyl conjugates by *A. lipoferum* has been reported by Piccoli *et al*. (1997), suggesting that the growth promotion in plants, which is induced by *Azospirillum* infection, may occur by a combination of both gibberellin production and deconjugation of plant-derived gibberellin-glucoside/glucosyl ester by the bacterium.

Exposure either to *Azospirillum* at a concentration of 10^9 cfu ml^{-1} (in the absence of *Rhizobium*) or to compounds excreted by the bacteria into the growth medium caused a 40% increase in endogenous ethylene production by the roots of burr medic (Yahalom *et al*., 1990). A less concentrated inoculum did not increase ethylene production. As IAA can stimulate ethylene production, it was suggested that the high exogenous IAA level probably caused an increase in the ethylene level in the plant.

P. putida GR12-2, on the other hand, is assumed to lower the level of ethylene in the developing plant (Glick, 1995). As mentioned earlier, this bacterium possesses the enzyme ACC deaminase (see section *2.4.*) that hydrolyses ACC, the immediate precursor of ethylene in plants. The presumed result of the hydrolysis of ACC is that the concentration of ethylene within the plant is lowered as a consequence of the increased exudation gradient of ACC (Glick *et al*., 1997). However, this hypothesis still needs to be confirmed by the measurement of ethylene levels in inoculated plants.

3.5. Mineral Uptake

Enhanced mineral uptake in plants inoculated with *Azospirillum* and other diazotrophs has been reported repeatedly, both in greenhouse experiments (Lin *et al*., 1983; Kapulnik *et al*., 1985a; Morgenstern and Okon, 1987b) and in the field (Sarig *et al*., 1984; 1988; Kapulnik *et al*., 1987; Fages, 1994). The major nutrient involved was fixed-nitrogen in the form of either nitrate in wheat, sorghum, corn and *S. bicolor x S. sudanense* plants (Lin *et al*., 1983; Kapulnik *et al*., 1985a; Pacovsky

et al., 1985; Morgenstern and Okon, 1987b; Boddey and Döbereiner, 1988; Kucey, 1988) or ammonium in rice plants (Murty and Ladha, 1988). Also, improved uptake of $H_2PO_4^-$ (Lin *et al*., 1983; Murty and Ladha, 1988; Sarig *et al*., 1988), K^+ (Lin *et al*., 1983), Rb^+ (Morgenstern and Okon, 1987b), and Fe^{2+} (Barton *et al*., 1986) by inoculated plants has been demonstrated. Likewise, inoculation of lowland rice with different rhizobia led to a significant increase in the uptake of both mineral N, P, and K by 10-28% and Fe^{2+} by 15-64% (Biswas *et al.*, 2000).

Even so, it is not yet generally accepted that this improved nutrient uptake is due to a specific enhancement of the normal ion-uptake mechanism. One rationale suggests that increased mineral uptake by inoculated plants is the consequence of a general increase in the volume and surface of the root system, as reflected by increased root-hair formation and increases in root number, thickness, and length (Reynders and Vlassak, 1982; Smith *et al*., 1984; Kapulnik *et al*., 1985a; 1987; Morgenstern and Okon, 1987b; Gunarto *et al*., 1999; Biswas *et al*., 2000). Higher K^+ and Fe^{2+} uptake, for instance, are related to thicker roots (Barber, 1985) and higher $H_2PO_4^-$ uptake to the presence of root hairs (Gahoonia and Nielsen, 1998; Gahoonia *et al*., 2001). Concomitant increases in root elongation, root surface area, and mineral uptake were indeed reported after inoculation of wheat with a mixture of *A. brasilense* strains (Kapulnik *et al*., 1985a, 1985b). Furthermore, using a hydroponic system containing NO_3^-, both the surface area of wheat roots and the uptake of NO_3^- from the mineral solution during plant growth were found to increase upon inoculation. However, no significant changes were obtained in the NO_3^- uptake/root-surface area ratio, indicating that the increased NO_3^- uptake by wheat inoculated with *Azospirillum* was due to general increase in root-surface area and not because of an increase in the specific uptake rate (Kapulnik *et al*., 1985a). Similarly, a significant correlation was found between root length and the level of labelled phosphorus (^{32}P) in roots of canola seedlings inoculated with *P. putida* GR12-2, indicating that it was the bacterial effect on root elongation that promoted the increase in the uptake of ^{32}P by the roots (Lifshitz *et al*., 1987).

An alternative rationale that involves the existence of direct effects on specific uptake mechanisms cannot be discounted. For example, *A. vinelandii* strain ATCC 12837 strongly enhanced both Ca^{2+} and Mg^{2+} concentrations in faba bean shoots co-inoculated with *Rhizobium*, but did not increase the concentration of other nutrients, like K^+, $H_2PO_4^-$, Fe^{2+}, $B(OH)_4^-$, Mn^{2+}, Zn^{2+} and Cu^{2+} (Rodelas *et al*., 1999). Murty and Ladha (1988) found enhanced NH_4^+ and $H_2PO_4^-$ uptake by rice plants inoculated with *Azospirillum* without a concomitant increase in the root-surface area and Lin *et al*. (1983) demonstrated that *Azospirillum*-inoculated corn and sorghum plants took up minerals (N, P and K) from solutions at faster rates than uninoculated controls.

With respect to this last result, inoculation with *A. brasilense* Cd was found to result in both a reduction in the membrane potential of the root cells of soybean seedlings and a significant increase in the proton efflux of the roots of wheat seedlings (Bashan *et al*., 1989; Bashan, 1991). These activities were proposed to be responsible for the increase in mineral uptake by *Azospirillum*-inoculated plants. Proton extrusion through the membranes of root cells, which results in acidification of the rhizosphere, is proposed to be a major mechanism in the mobilization of minerals by plants (Spanswick, 1981; Marschner *et al*., 1986). In hydroponic

systems, the enhanced specific uptake of minerals from the solution occurred concurrently with improved root hydraulic conductivity (25-40% increase as compared to controls) in sorghum roots (Sarig *et al*., 1992).

Alternatively, the surface activity involved in ion uptake may have increased as a result of the altered cell arrangements in the outer four or five layers of the root cortex as was observed in cross sections of inoculated plants (Lin *et al*., 1983; Kapulnik *et al*., 1985b; Yahalom *et al*., 1991). These morphological changes were found to have a physiological effect on inoculated roots; the altered area stained darker with methylene blue, suggesting an increase in the surface activity involved in ion uptake (Lin *et al*., 1983).

Recently, a gene encoding an ammonium transporter in the root hairs of tomato (*LEAMT1;2*) has been identified. Inoculation of N-depleted tomato plants with either *A. brasilense* or *Azoarcus* sp. induced *LEAMT1;2* expression, whereas *A. brasilense nifDK*$^{-}$ mutants failed to do so (Becker *et al*., 2002). This result indicates that NH_4^+, resulting from nitrogen fixation by the bacteria, might be used as a fixed-N source under these conditions. Alternatively, the transported NH_4^+ itself could represent a key signal in the associative interaction between higher plants and N_2-fixing microorganisms. Induction of ammonium transport in root hairs upon inoculation can lead to enhanced nutrient uptake by tomato roots and consequently promotion of plant growth.

3.6. Water Uptake

The larger root system of inoculated plants does not only lead to enhanced mineral uptake, it can also improve water uptake, which in turn could benefit crops growing in water-deficient soils. In field experiments, the water regime of sorghum plants was improved by inoculation as demonstrated by their higher leaf-water potential, lower canopy temperatures, and greater stomatal conductance and transpiration. Total extraction of soil moisture by inoculated plants was greater (by about 15 %) and occurred from deeper soil layers as compared with non-inoculated controls. These findings indicate that inoculation with *Azospirillum* could lead to yield increases in dry-land grain sorghum, primarily through improved utilization of soil moisture (Sarig *et al*., 1988; Fallik *et al*., 1994). Moreover, inoculation with *A. brasilense* was shown to significantly reduce the negative effects on plant growth caused by irrigation with saline water (Hamaoui *et al*., 2001). This reduction in salt stress could be attributed to the stimulation of root development and concomitant water uptake upon inoculation (Figure 6). Inoculation with *A. brasilense* also delayed leaf senescence in sorghum plants subjected to osmotic stress, indicating improved water uptake in the inoculated stressed roots (Sarig *et al*., 1990).

3.7. Nodule Function (BNF with Legumes)

Yahalom *et al*. (1987) studied the effect of combined inoculation with *Azospirillum* and *Rhizobium* on the acetylene-reduction activity (ARA) in pouch-grown burr

medic and siratro seedlings. In the case of burr medic, they found an average increase in the ARA of *ca*. 13-fold in the presence of *Azospirillum*, when compared with *Rhizobium* alone. Similarly, for siratro, ARA was increased by *ca*. 25% at day 13 after inoculation and by *ca*. 80 % at day 20 as compared to plants inoculated with *Rhizobium* alone. Likewise, a faster ^{15}N-dilution rate was measured in co-inoculated burr medic plants grown in pots as compared to those inoculated with *Rhizobium* alone (Yahalom *et al*., 1988). Further, combined inoculation of potted common-bean plants with *Rhizobium* and *Azospirillum* significantly increased N_2-fixation as reflected by the significantly higher ARA rates of co-inoculated plants compared with those inoculated with *Rhizobium* alone (Burdman *et al*., 1997).

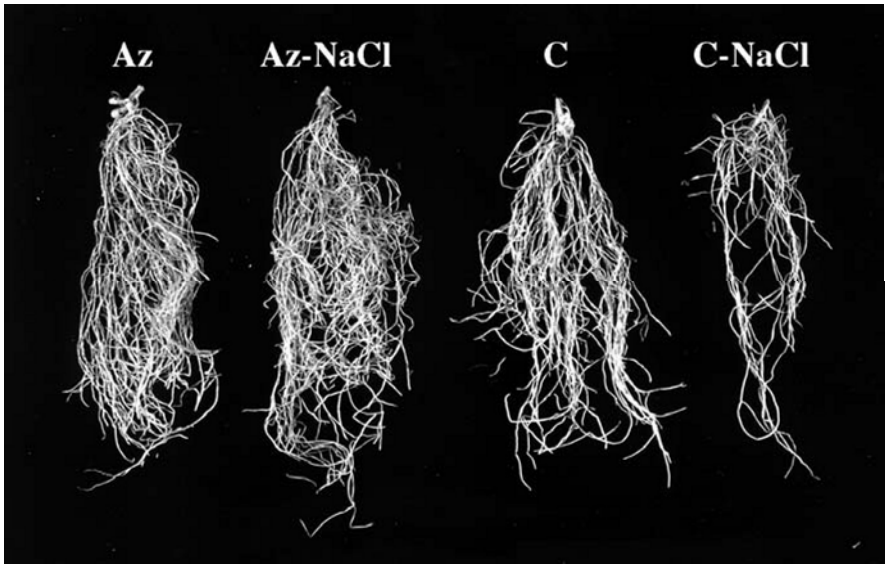

Figure 6. Effect of A. brasilense *strain Cd on root morphology and development of chickpeas in a greenhouse experiment 84 days after sowing.*
Az, root inoculated with Azospirillum *and irrigated with tap water; Az-NaCl,* Azospirillum *and saline water; C, non-inoculated and tap water; C-NaCl, non-inoculated and saline water. Reprinted from Hamaoui* et al*., 2001, with permission of EDP Sciences.*

4. EFFECTS ON PLANT GROWTH

4.1. Leaf Surface Area

Inoculation with *A. brasilense* of sorghum grown in hydroponic systems significantly enhanced leaf-area development mainly at 24-28 days after emergence (Sarig *et al*., 1990). At later stages, leaf senescence was delayed in inoculated plants, thus favouring dry-matter accumulation and grain filling.

4.2. Mineral Content

As mentioned earlier, inoculation with *Azospirillum* or other diazotrophs can result in an increase in the mineral uptake by plants. Whether this is due to the specific enhancement of the normal ion-uptake mechanism or to the promotion of root development, the net result is an increase in the mineral content of inoculated plants. Although increases in fixed-N content have been reported primarily, the content of other elements, like P and K, was also found to have increased (Sarig *et al.*, 1984; Yahalom *et al.*, 1984; Kapulnik *et al*., 1983; 1987; Boddey *et al*., 1986; Boddey and Döbereiner, 1988; Sumner, 1990). Inoculation of a local maize variety in India with strains of either *A. chroococcum* or *A. brasilense* resulted in significantly higher values for both the N and P contents of plant components (Pandey *et al*., 1998).

Also, co-inoculation of diazotrophs with rhizobia can affect the mineral content of inoculated plants. Combined inoculation of faba beans with *A. brasilense* and *R. leguminosarum* bv. viciae significantly increased the total N content of the co-inoculated plants at the flowering stage, when compared with plants inoculated with *Rhizobium* alone (Rodelas *et al*., 1996). In another study, mixed inoculations of faba beans with four different *Rhizobium/Azospirillum* and *Rhizobium/Azotobacter* combinations led to changes in total content, concentration, and/or distribution of the mineral macro- and micronutrients K, P, Ca, Mg, Fe, B, Mn, Zn and Cu, when compared with faba beans inoculated with *Rhizobium* alone (Rodelas *et al*., 1999). The effects varied greatly depending on the *Azotobacter* or *Azospirillum* strains selected for combined inoculation.

4.3. Carbohydrate Content

Inoculation with *Gluconacetobacter* (formerly *Acetobacter*) *diazotrophicus* promoted the accumulation of carbohydrates in the shoots of young sorghum plants (Bastian *et al*., 1999). Inoculated plants had larger amounts of both fructose and glucose than non-inoculated controls. It was already known with sugarcane that application of GA_3 significantly increased sucrose production (Nickell, 1988), however, with sorghum, hormonal treatments with GA_3 and IAA had only a moderate effect on its sugar content. The application of GA_3 did, however, increase the total carbohydrate, glucose, and fructose levels in sorghum shoots.

These results suggest that *G. diazotrophicus* acts only in part by producing hormones. The bacterium may also have other effects, like stimulating either invertase activity or another enzymatic system related to mono-sugar synthesis. It is also possible that the bacterium may have an indirect and more general effect on the whole plant; by providing hormones and reducing N_2, it could increase the overall growth and carbohydrate accumulation of the plant.

4.4. Water Status of the Plant

Inoculation of wheat with *A. brasilense* Sp245 improved coleoptile growth in seedlings that were grown in darkness under both osmotic and salt stress (Alvarez *et*

al., 1996; Creus *et al*., 1997). This stimulated growth was accompanied by significant decreases in osmotic potential and relative water content at zero turgor, in the volumetric cell-wall modulus of elasticity, and in absolute symplastic water volume and by a significant rise in apoplastic water-fraction parameters. These results are consistent with a better water status in *Azospirillum*-inoculated wheat seedlings under water stress, where effects on cell-wall elasticity and (or) apoplastic water were evident (Creus *et al*., 1998).

4.5. Timing of Flowering, Anthesis

Inoculation of tomato with *A. paspali* shortened the development time of the first and second trusses by 7 and 4 days, respectively, but the number of flower buds on both trusses was not affected (Barea and Brown, 1974).

Heading and flowering of foxtail millet in the field occurred earlier only in plants inoculated with *A. brasilense* and not in plants inoculated with *A. chroococcum* (Yahalom *et al*., 1984). In a greenhouse inoculation experiment with wheat, it was found that ears appeared earlier on plants inoculated with *Azospirillum* than on non-inoculated control plants (Dobbelaere *et al*., 2002).

4.6. Yield and Yield Components (mostly in the field)

Since the 1980's, the response of agriculturally important crops to inoculation with *Azospirillum* has been investigated in numerous field and greenhouse experiments carried out in various countries. The results from these field experiments were evaluated in several reviews (Bashan and Levanony, 1990; Sumner, 1990; Fages, 1994; Okon and Labandera-Gonzalez, 1994). Based on published data, it was concluded that inoculation with *Azospirillum* resulted in significant yield increases of the magnitude of 5-30% in about 60-70% of the experiments, often with even greater increases under greenhouse conditions (Sumner, 1990; Okon and Labandera-Gonzalez, 1994). The beneficial effects were mainly observed in lighter soils under intermediate levels of fertilizer (N, P and K) and water regimes (Okon and Labandera-Gonzalez, 1994). Less clear effects were generally observed in heavier soils and under high levels of fertilizer.

Part of this increased yield results from increases in the vegetative yield of inoculated plants. In sweet corn, there was an increase in top fresh weight and, in forage maize, there was an increase in the fresh- and dry-weight of leaves, stems, and percentage of leaves (Kapulnik *et al*., 1981). Inoculation of sorghum led to an increase in both fresh and dry weight of tops (Kapulnik *et al*., 1981). The improved final dry weight of inoculated plants was due to a 48% larger rate of accumulation during the period 40-70 days from emergence, which was due in turn to larger stem weights (Sarig *et al*., 1984). In foxtail millet and millet, there were significant increases in the weight of leaves and both the fresh and dry weight of tops (Kapulnik *et al*., 1981; Yahalom *et al*., 1984). Plant height was also significantly greater in inoculated plots (Kapulnik *et al*., 1981; 1982). In wheat, both plant height and the weight of all cultivars tested was increased (Kapulnik *et al*., 1983).

Apart from vegetative yield, inoculation with *Azospirillum* was also found to affect reproductive (grain) yield. Field inoculation of various maize cultivars with *A. brasilense* in different regions of Mexico showed consistent increases in grain yield (50% on average) under low levels of N fertilization (Dobbelaere *et al*., 2001). Earlier studies with sweet corn showed that the major factor contributing to increased yield was a significant increase in the average number of marketable ears per plant after inoculation with *Azospirillum* (Kapulnik *et al*., 1981; 1982). In sorghum, yield increases obtained after *Azospirillum* inoculation resulted from a significant increase in the number of panicles per plant, their weight, and the weight per 1000 seeds (Kapulnik *et al*., 1981; Sarig *et al*., 1984). In inoculation experiments with wheat, increases in grain yield were derived from increases in the number of fertile tillers per area unit (Kapulnik *et al*., 1983). In a recent field experiment carried out in Belgium, plants inoculated with *A. brasilense* had significantly heavier ears (up to 25% increase in dry weight) than the controls, due to the fact that on average they contained more grains that were significantly heavier (up to 20% increase in the grain dry weight for inoculated plants; Dobbelaere *et al*., 2001). *Azospirillum* inoculation was also beneficial to reproductive attributes of legumes; an increased grain yield of garden peas was due to a significant increase in the number of pods per plant (Sarig *et al*., 1986).

Other diazotrophs were also found to affect the yield of inoculated plants. *A. chroococcum* has been widely used to inoculate agricultural crops. Seed inoculation of non-legumes with *A. chroococcum* increases the yield of field crops by about 10% and of cereals by about 15-20% (Mishustin and Shilnekova, 1968; Hussain and Khan, 1973; Reddy *et al*., 1977; Singh *et al*., 1977; Hussain *et al*., 1987). Inoculation with *B. polymyxa* produced significant increases in both root and total plant biomass in white clover and crested wheatgrass (Holl *et al*., 1988). Antoun *et al*. (1998) tested 266 strains of rhizobia and bradyrhizobia for their PGPR effect on radish and found that 25% of all the strains tested stimulated radish dry matter yield (20% or more increase) in a greenhouse study.

5. FUTURE STUDIES

The effects of PGPR and *Azospirillum* on the morphology and physiology of plants has been studied within the confines of our current knowledge of root architecture, root function, plant-hormone metabolism, and many other related processes. Recently, there have been many scientific advances in our understanding of root morphology and physiology that will be used and will impact our understanding of the changes that take place in plants when they associate with PGPR (Waisel *et al*., 2002). Furthermore, the sequencing and evaluation of the entire genome of plants, such as rice, sugarcane, maize, and others, together with the use of modern techniques for high-throughput analysis (microarrays) for transcriptomics and proteomics, will detect changes in inoculated plants, *i.e*., PGPR-plant associations, faster and more accurately than is possible currently and will yield a vast amount of information on these interactions.

For the past 30 years, there has been an increasing effort in developing microbial inoculants (including rhizobia) for plant growth promotion in agriculture (Burdman *et al*., 2000; Dobbelaere *et al*., 2001; Walsh *et al*., 2001). Significant advances in the elucidation of the mechanisms involved in plant-growth promotion have been made, especially using molecular-biology approaches (Dobbelaere *et al*., 2003; Walsh *et al*., 2001). The mechanisms studies include mainly the role of biological nitrogen fixation, production of phytohormones, and biological control. Both the dynamics of colonization of plant surfaces by beneficial bacteria and rhizosphere ecology have received less attention. The various bacteria-plant systems have been mainly tested under controlled growth conditions and, in most cases, plant-growth promotion has been clearly demonstrated (Dobbelaere *et al*., 2001; Walsh *et al*., 2001). Much less testing has been performed under field conditions. One example is a field-based system that used *Azospirillum* inoculants. It showed significant crop benefits that were consistent for a number of experiments, which were carried out for several seasons and performed by personnel of either research institutions or of commercial-inoculant companies (Fages, 1994; Okon and Labandera-Gonzalez, 1994; Dobbelaere *et al*., 2001). In other cases, the researches have observed inconsistencies, especially after performing only limited field tests.

The scientific basis for using beneficial microbial inoculants must continue to be investigated and explored. There is a good possibility that the various systems developed will be used extensively in agriculture, particularly when practice demands a clear and substantial reduction of both fertilizer and pesticide use.

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Chapter 8

BIOCONTROL OF PLANT DISEASES BY ASSOCIATIVE AND ENDOPHYTIC NITROGEN-FIXING BACTERIA

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1. BENEFICIAL PLANT-ASSOCIATED NITROGEN-FIXING BACTERIA AND BIOCONTROL OF PLANT DISEASE

Soil microbial populations and communities associated with the roots of plants are affected both by the type of soil and by the plant (Latour *et al.,* 1996). This influence is more important in the zone of intense interaction between plant roots and soil bacteria called the rhizosphere (Hiltner, 1904). The rhizosphere is defined as a zone of high microbial activity that is driven in part by plant-root exudates (Bowen and Rovira, 1999) and is dependent on the plant species or cultivars as well as environmental factors. The microorganisms in the rhizosphere, stimulated by the plant-root exudates, can have either positive or negative effects (or none at all) on plant growth (Schippers *et al.,* 1987). The bacteria that stimulate plant growth are referred to as "plant growth-promoting rhizobacteria" (PGPR). Biocontrol agents can be defined as bacteria normally present (or introduced) in the rhizosphere that prevent the development of plant pathogens, so protecting plant health and preventing plant diseases.

In 1925, Beijerinck described the isolation of *Spirillum lipoferum*, which 50 later was recognized as the first "associative nitrogen-fixing bacterium", when the group led by Johanna Döbereiner reported the close association of this bacterium with grasses and cereal crops (Döbereiner and Day, 1976). Since then, an increasing number of nitrogen-fixing bacterial species have been discovered in association with non-leguminous plants; these include *Azospirillum* (formerly *Spirillum*),

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Azoarcus, Azotobacter, Beijerinckia, Burkholderia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Paenibacillus (formerly *Bacillus*)*, Pantoea agglomerans* (formerly *Enterobacter*)*, Phyllobacterium*, *Pseudomonas stutzeri*, and *Sphingomonas* (Bally *et al.,* 1983; 1990; Baldani *et al.,* 1986; Vermeiren *et al*., 1999; Tapia-Hernández *et al.,* 2000; Tran Van *et al.,* 2000; Gyaneshwar *et al*., 2001; Vargas *et al.,* 2003).

The role of associative nitrogen-fixing bacteria and more recently of nitrogen-fixing endophytes in plant-growth promotion is well established (see Chapters 5, 6, 7, 9, 10 and 13 of this volume). Plant-growth promotion depends on three different mechanisms: (i) some bacteria act as competitors of plant pathogens (Dobbelaere *et al.,* 2003); (ii) others directly stimulate root development through the production of phytohormones (Tien *et al.*, 1979; Barbieri *et al.,* 1986; Okon *et al.,* 1986; Fallik *et al.,* 1989; see Chapters 6 and 7 of this volume); and (iii) a third category produce a range of compounds that inhibit the proliferation of pathogens (Storm *et al.,* 1977; Tapia-Hernández *et al.,* 1990; Pichard *et al.,* 1995; Lebuhn *et al.,* 1997; Miché *et al.,* 2000; Lopez *et al.,* 2001).

In general, nitrogen-fixing PGPR are efficient root colonizers. The mechanisms of recognition and root colonization by soil microbes are documented in Chapters 4 and 5 of this volume. The increase in plant growth is due to complex factors, including mainly phytohormones production and to a lesser extent nitrogen fixation (Okon and Labandera-Gonzales, 1994; Dobbelaere *et al.,* 2003; see Chapters 6 and 7). Although several different mechanisms of biocontrol by PGPR have been studied (reviewed by Handelsman and Stabb, 1996; Bloemberg and Lugtenberg, 2001; Dobbelaere *et al.,* 2003), little is known as yet concerning the biocontrol exerted by nitrogen-fixing bacteria when colonizing the rhizosphere, merely because the ability of biocontrol bacteria to fix nitrogen has not been fully explored. In addition, some nitrogen-fixing bacteria can either play a direct role as antagonists against pathogenic microorganisms or induce "systemic resistance" (see section 5). This protection even applies toward parasites (Chanway, 1998).

Three levels of interaction will be discussed, with an emphasis on the data obtained with nitrogen-fixing bacteria. First, in terms of population dynamics, the rhizospheric community structure can be altered by the interaction of the biocontrol bacterial population with the components of the microbial community present in the rhizospheric soil. Second, biocontrol bacteria can interact directly with the plant pathogen whether it is bacterial, fungal, animal or parasitic plant. Third and finally, biocontrol bacteria may modify the plant's susceptibility to pathogen attack through direct interactions with the host plant.

2. INTERACTIONS WITHIN MICROBIAL COMMUNITIES: COMPETITION MECHANISMS

2.1. Soil Structure and Properties and Evolution of Microbial Communities

Bacteria acting as biocontrol agents can be both sources and sinks for plant nutrients. They can either influence or be influenced by other bacteria and fungi in the surrounding microbial community. Their activity and numbers are in part a

function of the soil characteristics (Chaussod *et al.*, 1986; Laad and Foster, 1987; Amato and Ladd, 1992). The effects of soil structure on the location and turnover of microbial populations and communities in soil have been studied (Elliot, 1986; Jocteur-Monrozier *et al.,* 1991; Chotte *et al.*, 2002). For example, a microbial community colonizing particles of residual organic matter can lead to the release of degradation products. Then, the soluble organic compounds that diffuse into soil aggregates can be degraded by microorganisms located within the silt-sized micro-aggregates resulting in modification of the bacterial communities (Chotte *et al.,* 1998). Elo *et al.* (2000), in studying the pioneering vegetation after clear-cutting of the coniferous forest in Norway, analyzed the potential of an acidic spruce humus (pH 3.7-5.3) layer to supply beneficial rhizobacteria to birch and alder trees and to fescue grass. The humus contained significant quantities of both nitrogen-fixers and bacteria antagonistic to plant pathogens. The proliferation of these bacteria was stimulated in the rhizosphere, so modifying the microbial community (Elo *et al*., 2000).

2.2. Microbial Population Dynamics

The principal process of interaction within the soil microbial community is competition for both space and nutrients. The competition for space is, thus, dependent on abiotic factors in addition to biological ones. The abiotic factors include soil structure, texture, and pH (Landa *et al.*, 2001). The biotic factors involve competition during surface colonization and biofilm formation (Benizri *et al.*, 2001; Möenne-Loccoz *et al*., 2001). Competition can lead to a variety of subsidiary interactions, such as either synergistic or reduction-suppression with microbial-population movements.

2.2.1. Synergy

Synergistic interactions among bacteria that are involved in either biocontrol or plant-growth promotion and in association with nitrogen-fixing bacteria can lead to an increase of the soil microbial community responsible for the inhibition or suppression of plant pathogens (Glick, 1995; Kloepper, 1993; Handelsman and Stabb, 1996). This suppressive effect is stimulated and amplified in the rhizosphere as compared to the bulk soil (Kloepper, 1993). Several mechanisms have been proposed to account for the synergy effect, in particular thwarting plant pathogens, phosphate solubilization (Alagawadi, 1992; Belimov, 1995; Muthukumar, 2001; Rojas, 2001), iron sequestration, and antibiotic production (Whipps, 2001).

Associations between nitrogen fixers (such as *Azospirillum* and *Phyllobacterium*), biocontrol bacteria (*Pseudomonas*), and plant growth-promoting agents (VAM fungi and *Bacillus licheniformis,* both of which solubilize phosphate) have been demonstrated to affect plant pathogens. For example, the association of *Azospirillum* with a *Pseudomonas* during mycorrhizal colonization of a maize rhizosphere that had been inoculated with *Glomus mosseae* and *Glomus desertificalis* led to both a stimulation of plant growth and changes in the soil microbial community, especially to a decrease of plant pathogens (Vazquez *et al.*,

2000). Further, a synergistic relationship was observed between strains of *Phyllobacterium* and *Bacillus licheniformis* in a semi-arid Mangrove rhizosphere (Rojas *et al*., 2001). Increases in both nitrogen fixation and phosphate solubilization occurred after inoculation with the two bacteria as compared to the effects observed when cultures were added individually. A synergistic effect in the suppression of the root-rot disease of cotton caused by *Rhizoctonia bataticola* has also been observed during the combined application of *Azospirillum* and *Pseudomonas fluorescens* (Marimuthu *et al*., 2002).

2.2.2. Reduction of Pathogenic Populations

The second phenomenon is the reduction (or suppression) of pathogenic populations due to their displacement (and sometimes replacement) by other non-pathogenic microorganisms (Murphy *et al.,* 2000; Mazzola, 2002). Although this mechanism is well known for plant growth-promoting rhizobacteria, little is known about the involvement of nitrogen fixers as yet. A well-known example is *P. fluorescens,* which has a suppressive effect on disease caused by *Fusarium* by displacing the pathogenic fungal population and aiding their replacement by the non-pathogenic fungus *Fusarium oxysporum* (Schippers *et al*., 1987). Another example is the application of *Pantoea agglomerans,* which is antagonistic toward the plant pathogen *Pseudomonas syringae* the agent of basal barley kernel blight. The percentage of infected kernels decreased when *P. agglomerans* was applied before the pathogen was inoculated (Braun-Kiewnick *et al.,* 2000), suggesting that the occupation of the ecological space was one of the important characteristics of the biocontrol agent's efficiency.

3. BIOLOGICAL CONTROL AGAINST SOIL-BORNE DISEASES

3.1. Interaction with Plant Pathogens: Competition for Nutrients

One of the mechanisms of interaction between nitrogen fixers and plant pathogens is the competition for nutrients and oligo-elements in the rhizosphere. Plant exudates represent an important source of nutrients for soil microorganisms. However, due to a high C/N ratio and to limiting concentrations of iron and phosphorus, the exudates encourage the proliferation of some bacterial species over other microorganisms. This competition can lead to microbial population displacement and even disappearance (Duijff *et al.,* 1993; Handelsman and Stabb, 1996; Dobbeleare *et al.,* 2003). It also often leads to an increase in the population of biocontrol agents and a concomitant decrease in plant pathogen numbers. Although nitrogen-fixing bacteria are sometimes known for their capability to suppress plant pathogens, the mechanisms involved are not always well understood. Some of them, such as *Azospirillum, Azotobacter,* and *Klebsiella*, limit the development of soil-borne plant pathogens, such as *Fusarium oxysporum, Rhizoctonia solani, Sclerotinia*, and *Pythium* sp., which attacks cucumber (for a review, see Raupach and Kloepper, 1998). Often, several mechanisms, such as iron sequestration (*Azospirillum, Azotobacter, Pseudomonas*), phosphate solubilization (*Azospirillum, Azotobacter, Gluconacetobacter, Pseudomonas*), antibiotic

production, enzyme inactivation, etc., are suspected to be involved. Bashan and Bashan (2002) demonstrated that the reduction of the tomato pathogen, *Pseudomonas syringae* pv. tomato, was the result of the combination of *A. brasilense* application and ~~s~~treptomycin sulphate and chemo-thermal seed treatment.

3.2. Competition for Iron

Iron is an essential nutrient that is required for a variety of functions including formation of heme. Competition for iron in the rhizosphere is greater than that in the bulk soil. In aerobic environments, iron is converted to oxyhydroxide polymers and, due to their relative insolubility, iron mobilization requires specific chelators, such as siderophores (Romheld and Marschner, 1990). Siderophores are low molecular-weight Fe^{3+}-specific chelator molecules, which act as iron-scavenging agents. Thus, to some extent, they may be antibiotics or antimicrobial agents.

Most siderophores are either hydroxamates or catechols. They are produced by many microorganisms in response to iron deficiency in the environment (Neilands, 1995). Aerobactin was the first reported siderophore; it was isolated from *Aerobacter aerogenes* (now *Enterobacter*) in 1969 (see Neilands 1995). The study of the genetics of iron transport in enteric bacteria led to the identification of the *fur* gene (ferric uptake system) that controls aerobactin synthesis and a set of genes that encode membrane-bound proteins belonging to the *fur* regulon (Neilands 1995). Production of siderophores in pathogens is a trait often associated with bacterial virulence. *Pseudomonas aeruginosa* and the related fluorescent *Pseudomonas* produce efficient iron scavengers called pyoverdines or pseudobactins. Synthesis and regulation of chrysobactin, a catechol-type siderophore produced by the plant pathogen *Erwinia chrysanthemi* responsible of the soft-rot disease, was recently reviewed (Expert, 1999).

Among beneficial bacteria, diazotrophs such as *A. brasilense*, *A. lipoferum,* and *Azotobacter vinelandii*, which are known to have strong affinities for Fe^{3+}, are strongly stimulated in the rhizosphere (Bally *et al.*, 1983; Döbereiner and Pedrosa, 1987). They produce various siderophores that sequester iron and reduce its availability to other microorganisms, including pathogens (Saxena *et al.,* 1986; 1989; Shah *et al.,* 1992). Production of phenolate-type siderophores, derivatives of dihydroxybenzoic acid, occurs in *Azotobacter* (azotochelin and aminochelin) and *Azospirillum* (spirillobactin) and production of salicylic acid was reported in *A. lipoferum* and *Burkholderia* spp. (Page and von Tigerstrom, 1988; Saxena *et al.,* 1986; Parke and Gurian-Sherman, 2001). Thus, these nitrogen-fixing bacteria are potential biocontrol agents, however, to date, siderophore production has not been demonstrated for these bacteria in the soil.

3.3. Antimicrobial Agents

An antagonistic mechanism involves the production of anti-fungal metabolites (AFMs) and antibiotics as well as siderophores (see section 3.2) by bacteria in the

rhizosphere (Whipps, 2001). To remain competitive, PGPR and/or nitrogen-fixing bacteria must be able to produce a wide range of metabolites (Wood and Pierson, 1996) and they must also possess mechanisms of resistance to these metabolites.

3.3.1. Antifungal Metabolites and Antibiotic Production in P. fluorescens

The main AFMs and antibiotics with biocontrol properties in fluorescent *Pseudomonas* include phenazines (phenazine-1-carboxylate, pyocyanine), pyrrolnitrin, phloroglucinols (2-4-diacetylphloroglucinol - DAPG), pyoluteorin, lipopeptides (viscosinamide), and HCN (Raaijmakers *et al*., 2002). These compounds are active against a range of bacteria, fungi and nematodes (Cronin *et al*., 1997b; Raaijmakers *et al*., 2002; Ramette *et al*., 2003). For example, pyrrolnitrin has a very wide host range and is active against several fungal pathogens, such as *Rhizoctonia solani*, *Botrytis cinerea*, *Verticillium dahliae* and *Sclerotinia sclerotiorum*. Production of the antifungal and antimicrobial agent DAPG is essentially limited to *Pseudomonas* (Picard *et al*., 2000; Wang *et al*., 2001), whereas synthesis of pyrrolnitrin also occurs in *Burkholderia*, *Pantoea* and *Serratia* (Raaijmakers *et al*., 2002). Of interest, some nitrogen-fixing strains have been identified among members of these later genera (see Chapters 2 and 3).

The chemical structures of *P. fluorescens* AFMs are depicted in the recent review of Haas and Keel (2003) and the pathways for their synthesis are known (see Pierson and Tomashow, 1992; Bangera and Thomashow, 1999; Bloemberg and Lugtenberg, 2001; Whipps, 2001; and refs. therein). Importantly, the metabolites produced by *Pseudomonas* absorb to organic soil particles and minerals and they can be detected in the rhizosphere. Characterization of the antimicrobial activities derives from *in vitro* assays and also from the isolation of bacterial mutants defective in the production of these metabolites (reviewed by Haas and Keel, 2003).

For example, Tn*5*-insertion mutants in the *phz* locus, encoding the synthesis of phenazine-1-carboxylate, led to mutant strains with decreased ability to suppress a major wheat-root disease, the "take-all", caused by the fungal pathogen *Gaeumannomyces graminis* var. tritici (Tomashow and Weller, 1988). Manipulation of the structural genes for the synthesis of diverse AFMs has led to the construction of recombinant strains. Introduction of the *phz* locus (*phzABCDEFG* operon) into the chromosome of a *P. fluorescens* PGPR strain resulted in an increased ability to reduce damping-off disease of pea seedlings caused by *Pythium ultimum* (Timms-Wilson *et al*., 2000). However, stable introduction of either the *phz* operon into a DAPG-producing strain or of the *phl* operon (coding for DAPG) into a phenazine-producing strain did not enable the recombinant strains to compete with the indigenous soil microflora (Viebahn *et al*., 2003).

3.3.2. Antimicrobial-Agent Production in Associative and Endophytic Diazotrophs

Many nitrogen fixers, such as *Azotobacter*, *Azospirillum*, *Burkholderia, and Paenibacillus*, produce AFMs and antibiotics that inhibit the plant pathogens associated with the same host plants. However, little is known regarding either the genetics and pathways of synthesis of these molecules or the regulation of their mechanisms of synthesis. For example, *Azotobacter* produces a low molecular-

weight antifungal compound that inhibits the production of conidia from *Botrytis* (Doneche and Marcantoni, 1992). *Paenibacillus polymyxa* produces polymyxine, an antibiotic active against several fungi and bacterial pathogens (Pichard *et al.,* 1995; Storm *et al.,* 1977). In addition to siderophores, *A. brasilense* and *A. lipoferum* strains are also able to produce bacteriocins (Oliveira and Drozdowicz, 1988; Tapia-Hernández *et al.,* 1990), however, their production has not been demonstrated in the soil.

The case of the *Burkholderia cepacia* complex is of particular interest because it comprises very diverse strains that were isolated from soil, rhizosphere, and hospital environments. Some isolates are plant or human pathogens, some display biocontrol activity with the production of both AFMs and siderophores, and others can enhance crop productivity (Parke and Gurian-Sherman, 2001; see Chapter 2). This is certainly the situation with *Burkholderia vietnamiensis*, an endophyte of rice. *B. vietnamiensis* inoculation of rice significantly increases crop yields (Tran Van *et al.,* 2000); it also produces several antibiotics (Miché *et al.,* 2000).

3.4. Hydrolytic Activities

Many soil bacteria, including free-living nitrogen-fixing bacteria, produce hydrolytic enzymes (chitinases, proteases) that allow them to act directly on plant pathogens. These bacteria are found throughout the rhizosphere and act mainly on fungal pathogens and parasitic nematodes (Cronin *et al*., 1997a; Dunne *et al*., 2000).

Chitin, an insoluble linear polymer consisting of β-1,4- *N*-acetylglucosamine units, is a major constituent of cell walls of most fungi. The chitinolytic enzyme complex from a *P. agglomerans* strain has been characterized (Chernin *et al*., 1995). The *chiA* gene encodes an endochitinase, which catalyzed random hydrolysis of 1,4-β linkages within the chitin molecule. It was cloned and introduced into *E coli*. This endochitinase exhibited antifungal activity and the recombinant *E. coli* strain prevented the root-rot disease caused by the fungus *Rhizoctonia solani* in cotton seedlings (Chernin *et al*., 1995; 1997). The nitrogen-fixing bacterium *Paenibacillus polymyxa* isolated from a wheat rhizosphere (Mavingui *et al.,* 1992) was found to exhibit chitinase activity as well as antifungal activity against the wheat pathogen *G. graminis* var. tritici (Mavingui and Heulin, 1994). *Gluconacetobacter diazotrophicus* inhibits the growth of the sugarcane pathogen *Xanthomonas albilineans* due to the production of a cell-wall lytic enzyme with lysozyme-like activity (Pinon *et al*., 2002; see Chapter 10).

Hydrolytic activities, which may be important during the colonization of the root surface and root interior by associative and endophytic nitrogen-fixing bacteria, have been described. Glucanase activities have been observed in *Klebsiella* and *Azoarcus* (Reinhold-Hurek *et al*., 1993; see Chapter 5 and 9 of this volume), whereas *Azospirillum irakense* displayed pectinolytic activity (Khammas and Kaiser 1991) and was also found to produce cellobiohydrolases and aryl-β-glucosidases (Faure *et al*., 1999). Further information on some of the genes coding for these enzymes is reviewed in Chapter 5 of this volume.

3.5. Parasitism of Pathogens

In addition to the mechanisms discussed above, some microorganisms can reduce or even eliminate plant pathogens by parasitic action against the pathogen. A classic example is the action of the Trichodermes (Chet *et al.,* 1981). *Trichoderma* spp. are parasites of fungal plant pathogens (Goldman *et al.,* 1994). This interaction requires specific reactions based upon the relationship between a parasite and its host, including the production of specific enzymes, notably lytic enzymes. *Bdellovibrio* have been also isolated as endoparasites of phytopathogenic bacteria (Jurkevitch *et al*., 2000)

3.6. Protection towards Plant Parasites

Among nitrogen-fixing bacteria, *A. brasilense* L4 is the best example of a biocontrol agent exerting its action against the parasite *Striga* (*Scrophulariaceae*) (Kabir *et al.,* 1996). *Striga* spp. are obligate parasitic weeds of tropical cereals that most often have the same host plant as *Azospirillum* (Bouillant *et al.,* 1997*).* Miché *et al.* (2000) have shown that *A. brasilense* prevents *Striga* seed germination by producing small lipophilic compounds.

4. REGULATION OF BIOCONTROL PROPERTIES AND CELL-CELL COMMUNICATION

In general, both secondary metabolite (AFMs, antibiotics, siderophores) and exoenzyme production occur at optimal level when the producing bacteria reach the high cell densities favourable for the production of quorum-sensing molecules (Larkin and Fravel, 1988; Fuqua *et al*, 1994). Regulatory networks that control global changes in gene expression (global regulation) and quorum sensing are the key mechanisms in the regulation of the biosynthesis of AFMs (Bloemberg and Lugtenberg, 2001; Haas and Keel, 2003; Figure 1).

4.1. The GacA/GacS Signal-Transduction Pathway

Global regulation of this pathway depends on the genes *gac*S/*gac*A, which encode a two-component regulatory system (Haas and Keel, 2003). GacS (LemA), initially found in the plant pathogen *Pseudomonas syringae* pv. *syringae,* is a trans-membrane histidine kinase, which senses a still-unknown signal and activates by phosphorylation the response regulator GacA, which is a member of the FixJ family (reviewed by Heeb and Haas, 2001). The *gacA/gacS* genes occur in several plant and animal pathogens as well as in biocontrol strains. They occur in strains of *Pseudomonas aeruginosa* (Parkins *et al*., 2001) and several other *Pseudomonas* species (Chatterjee *et al*., 2003), in *Azotobacter vinelandii* (Castañeda *et al*., 2000; 2001) and other members of γ–Proteobacteria (*e.g., Escherichia coli* as BarA/UvrY), in *Pectobacterium carotovorum* (formerly *Erwinia carotovora* as ExpS /ExpA), and in *Vibrio cholerae* and *Salmonella enterica* (as BarA/SirA) (Heeb and Haas, 2001). A recent report, based on PCR amplification of *gacA* in

environmental and plant samples, revealed the presence of these genes among members of β–Proteobacteria (in *Burkholderia* and *Comamonas*) also (de Souza *et al.*, 2003).

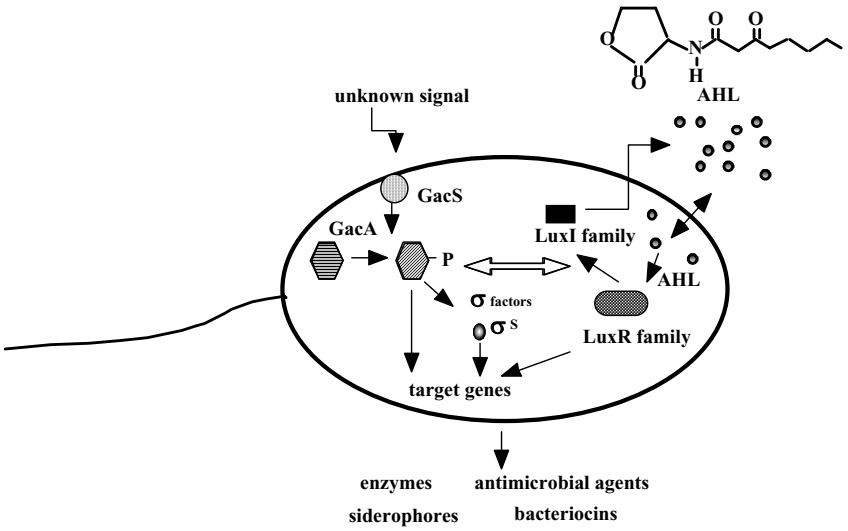

Figure 1. Schematic representation of some regulatory elements identified as important in the biocontrol of plant diseases in Gram-negative bacteria. GacS/GacA global regulation is essentially found among γ–Proteobacteria and some members of the β-subgroup. Gene regulation mediated by quorum-sensing molecules (AHLs) is wider in range and is found among the α–, β– and γ–subgroups of Proteobacteria. The hierarchy between GacA, quorum sensing, and sigma factors (RpoS, RpoN) differs in bacteria. In P. aeruginosa*,* P. chlororaphis *and* P. aureofaciens*, the GacS/A system controls the production of secondary metabolites and enzymes through the regulation of the quorum-sensing machinery. In* P. fluorescens *CHAO, AHL-signal molecules have not been detected, thus, secondary-metabolite production is under the control of the GacS/GacA system. The chemical structure of the 3-oxo-hexanoyl-homoserine lactone AHL is shown. See text for examples and references.*

The GacS/GacA signal-transduction regulatory cascade operates like a switch between primary and secondary metabolism. It controls the synthesis of DAPG (*phl* genes), pyoluteorin (*plt* genes), and HCN (*hcn* genes) in *P. fluorescens* CHA0 (Figure 1). Thus, *gacA* mutants displayed reduced biocontrol activity on fungal pathogens. The sigma factors, RpoD (σ^{70}) and RpoS (σ^{S}, stationary phase), are also involved in the regulation of antibiotic production in *P. fluorescens* because GacA/GacS influence *rpoS* transcription and σ^{S} accumulation (Whistler *et al*., 1998; Haas and Keel, 2003). The GacS/GacA regulatory cascade elucidated in *P. fluorescens* involves regulatory untranslated RNA molecules, *rsmZ* and *rsmY*

(Valverde *et al*., 2003). In its phophorylated form, GacA activates the transcription of *rsmZ* and *rsmY*. These RNA molecules are believed to complex the RNA-binding proteins RsmA and RsmE, which negatively control the expression of the *phl, plt* and *hcn* operons. A similar system that involves the titration of RNA-binding repressor molecules has been described in *P. carotovorum* and *E. coli* (Haas and Keel, 2003) and in *P. syringae* pv. tomato (Chatterjee *et al*., 2003).

Mutation in *gacA* in the pathogens *P. aeruginosa* and *P. syringae* led to strains impaired in pathogenicity, but in different functions. GacA controlled the production of virulence factors, pyocyanine, HCN, and lipase, in *P. aeruginosa* as well as the production of N-butyryl-homoserine lactone and biofilm formation (Reimmann *et al*., 1997; Parkins *et al*., 2001). In *P. syringae* pv. tomato, mutation in the *gacA* gene resulted in strains with a reduced level of expression of several sigma factors, RpoN, RpoS and HprL (type III secretion), with reduced levels in regulatory RNA (*rsmZ/ rsmB*), and impaired in the capacity to elicit the hypersensitive response (Chatterjee *et al*., 2003). GacA was found to control alginate and poly-β-hydroxybutyrate production in *A. vinelandii* and to be required for the production of RpoS (Castañeda *et al*., 2001). A compilation of the functions controlled by GacS/GacA is reported in the review of Heeb and Haas (2001).

4.2. Quorum-Sensing Regulation

Quorum-sensing regulation was discovered in the 1970s in luminescent marine *Vibrio* (reviewed by Fuqua *et al.*, 1994). In Gram-negative bacteria, most quorum-sensing systems consist of a transcriptional activator of the LuxR family and a signal molecule that plays the role of auto-inducer. The synthesis of N-acyl-homoserine lactone (AHLs; Figure 1) signal molecules depends of an auto-inducer synthase of the LuxI family (Fuqua *et al.*, 1994). The concentration of the auto-inducer is critical and it increases with cell density until it reaches a threshold concentration, where it binds to the transcriptional activator, which then activates transcription of target genes (Figure 1).

Quorum sensing in Gram-positive bacteria is mediated by peptides and depends on a two-component system, a sensor kinase and a response regulator (reviewed by Miller and Bassler, 2001). With time, an increasing number of functions are recognized as regulated by quorum sensing; these include bioluminescence, virulence factors, conjugation, motility and surface translocation, production of secondary metabolites production, exoenzymes, *etc*.

Besides *Vibrio* species, quorum-sensing systems have been described in *Agrobacterium tumefaciens* (TraI/TraR*), P. carotovorum* (ExpI/ExpR), *Pseudomonas,* and several rhizobia (Fuqua *et al*, 1994; Eberl, 1999; Miller and Bassler, 2001; Gonzalez and Marketon 2003). A large number of LuxR and LuxI homologues have been found within the α–, β– and γ–subclasses of Proteobacteria. Multiple homologues are found in some species, for example, *P. aeruginosa* carries two pairs, LasI/LasR and RhlI/RhlR, as well as a third LuxR homologue, Qsr (Chugani *et al*., 2001). A phylogenetic analysis revealed that some *luxI/luxR*

homologues might have been acquired by horizontal transfer in some species (Gray and Carey 2001).

High concentrations of bacteria in the rhizosphere are favourable for the production of quorum-sensing molecules. Indeed, production of AHL-signal molecules by Gram-negative bacteria is commonly found when in symbiotic or pathogenic association with plants (Cha *et al*., 1998; Elasri *et al*., 2001). Recently, bacteria capable of degrading AHLs have been identified in the rhizosphere; these may play a crucial role in pathogenicity or in biocontrol efficiency (Molina *et al*., 2003; Uroz *et al.*, 2003).

The production of several AFMs, in particular phenazines, as well as enzymes is quorum-sensing dependent (Wood *et al.,* 1997; Bloemberg and Lugtenberg, 2001; Haas *et al*., 2002). Phenazine-antibiotic production in two biocontrol strains, *Pseudomonas aureofasciens* and *Pseudomonas chlororaphis*, is under the control of the LuxI/LuxR homologue pair, PhzI/PhzR. Expression of *phzR* itself is controlled by the GacA/GacS system (see review by Haas *et al*., 2002), which accounts for the fact that *gacA* mutants are strongly impaired in their biocontrol properties. A hierarchical quorum-sensing cascade controls virulence factors and secondary-metabolite production in *P. aeruginosa* because LasR controls *rpoS* and *rhlR* expression (Latifi, *et al*., 1996). GacA positively controls the production of quorum-sensing signals and hence the regulatory quorum-sensing cascade (Reimmann *et al.*, 1997), whereas QscR, which acts as a repressor of *lasI* and *rhlI*, downregulates the production of quorum-sensing-dependent virulence factors (Chugani *et al*., 2001).

Among nitrogen fixers, quorum-sensing regulation was first studied in *Rhizobium leguminosarum* (for a review, see Wisniewski-Dye and Downie, 2002). Since then, it has been discovered in *Rhodobacter sphaeroides*, *Sinorhizobium*, *Rhizobium etli*, and *Bradyrhizobium japonicum* (Miller and Bassler, 2001; González and Marketon, 2003). For a long time, *R. leguminosarum* strains were known to produce plasmid-borne bacteriocins. Now, the chemical nature of the *small* bacteriocin has been characterized and shown to be an AHL-signal molecule, an *N*-(3-hydroxy-7-*cis*-tetradecenoyl)-L-homoserine lactone (Gray *et al*., 1996; Schripsema *et al*., 1996). In addition, four *luxI/luxR* pair homologues, called *rhiI/rhiR, cinI/cinR, traI/traR*, and *raiI/raiR*, have been subsequently characterized to make *R. leguminosarum* one of the most sophisticated quorum-sensing regulation systems known to date (Rodelas *et al*., 1999; Wisniewski-Dye and Downie, 2002; González and Marketon, 2003).

There are no reports of quorum-sensing regulation in associative and endophytic nitrogen fixers. However, it has been observed in *Burkholderia* (CepI/CepR) and *Pseudomonas*, both of which contain nitrogen fixers, and for *Rhodobacter* and several rhizobia. Thus, cell-density regulation is likely to play a role in other nitrogen-fixing members of α–Proteobacteria. Cell-cell signalling might be important during either surface or endophytic colonization. Flocculation (described in Chapter 5 of this volume) with *A. brasilense* appears as a process susceptible for triggering by cell-density regulation, although this is not proven. FlcA, a transcription regulator of the LuxR/UhpA family, controls flocculation and capsule

formation in strain *A. brasilense* Sp7 but, to date, no gene encoding a LuxI homologue has been found in this particular strain (Pereg-Gerk *et al*., 1998).

5. PLANT RESPONSE TO PATHOGENS AND BIOLOGICAL CONTROL IN THE RHIZOSPHERE

5.1. Plant Systemic Acquired Resistance

Activation of plant defences, when the plant is under pathogenic attack, is well documented (for a review, see Hunt and Ryals, 1996; Delaney 1997; Mauch-Mani and Métraux 1998). The plant response, called pathogen induced "systemic acquired resistance" or SAR, affects the entire plant. This pathway, induced after a pathogen attack, results in significant reduction of the disease symptoms. This plant response, identified initially in tobacco, has been observed in monocot species, including barley, wheat and maize. SAR is associated with the expression of a set of genes and with dramatic increases in the level of salicylic acid in both infected and non-infected tissues of the plant (Ryals *et al*., 1996). Salicylic acid is thought to play a role in the SAR-signalling pathway. This assumption is based on the properties of transgenic plants expressing the bacterial gene *nahG* that encodes for the enzyme salicylate hydroxylase. This enzyme converts salicylic acid to catechol and, thus, prevents the accumulation of salicylic acid in the plant, resulting in the absence of the SAR response to infection. Delaney (1997) demonstrated that another signal was also involved.

5.2. Induced Systemic Resistance

The colonization of pathogen-infected plant roots by saprophytic bacteria can also induce a pathogen-resistance mechanism in the plant. These saprophytic bacteria thus reduce the plant-disease symptoms within the host plant. This mechanism is called "rhizobacteria-mediated induced systemic resistance" or ISR. Jasmonic acid- and ethylene-signalling pathways are involved in this mechanism (Pieterse *et al.,* 2000). This mechanism is less efficient than that due to SAR. Persello-Cartiaux *et al.* (2003) proposed that non-pathogenic bacteria could be involved in a jasmonic acid- and ethylene-associated resistance as well as that of a salicylic acid-independent pathway. This latter pathway is complicated by the identification of non-pathogenic rhizospheric bacteria that favour the production of proteins via the salicylic acid-independent pathway. The SAR and the ISR responses enable plants, or at least plant tissues not yet infected, to develop a much greater resistance to the plant pathogen as well as to other pathogens.

5.3. Induced Systemic Resistance and Associative Nitrogen-Fixing Bacteria

Studies concerning ISR have dealt to date with dicotyledons and a similar system is still unknown for the monocotyledons (Dobbeleare, 2003). Furthermore, it concerns a relatively limited number of inducing bacterial species, mainly belonging to *Pseudomonas* PGPR. The two systems, SAR and ISR, can provide

much greater plant protection together than when alone, so suggesting that there might be some additive or synergistic effect (van Wees *et al.,* 2000). The only example of a nitrogen-fixing bacterium involved in ISR is that of *P. polymyxa,* which is known to induce plant resistance to the pathogen *P. carotovorum* (Timmusk and Wagner, 1999). This bacterium induces a range of biological stresses, which provoke a systemic response against the pathogen, involving the jasmonic acid, ethylene, and PR-1 pathways (Berger *et al.,* 1996).

6. CONCLUDING REMARKS

Although the application of biocontrol bacteria in the field still produces inconsistent results, the control of soil-borne pathogens by biological means rather than by agrochemical usage represents a challenge for this new Century in order to protect the environment and to develop sustainable agriculture (Paulitz and Bélanger, 2001). A certain number of plant disease-suppressive microorganisms have been commercialized; they include formulations containing *Pseudomonas, Bacillus*, and *Streptomyces* and among fungi, *Trichoderma* and non-pathogenic *Fusarium* (Paulitz and Bélanger, 2001).

Fundamental knowledge of the synthesis and the regulation of antifungal metabolites and other antibiotics is extensive for the fluorescent *Pseudomonas* (Haas and Keel, 2003). Indeed, the range of compounds produced by and the number of target pathogens for *P. fluorescens* strains are both large (Raaijmakers *et al*., 2002). In addition, it is striking to consider that many genes, which regulate biocontrol properties, are also involved in the control of virulence in pathogens. Although the information is still limited for nitrogen fixers, examples reported here for *Azospirillum, Azotobacter, Burkholderia, Klebsiella, Gluconacetobacter, Paenibacillus*, and *Pantoea s*uggest that, in addition to both phytohormone production and nitrogen fixation, biocontrol properties may account for the plant-growth promotion effect reported.

Further extensive screening of biocontrol properties is needed, together with the chemical characterization of the biological agents and the biochemical and genetic characterization of the synthesis pathways involved. In particular, the production of quorum-sensing signals needs to be explored as well as the presence of regulatory genes similar to those involved in the GacS/GacA-transduction pathway. Available techniques that are able to localize and follow the fate of bacterial populations should give more information on the dynamics of the nitrogen-fixer populations with respect to their ability for *in situ* production of putative antifungal metabolites and siderophores.

As several biocontrol operons for antifungal metabolites from *Pseudomonas* and some other bacteria have been cloned (Raaijmakers *et al.*, 2002), it may be of interest to transfer the biocontrol properties to nitrogen fixing strains, in particular to endophytes, to assay their suppressive properties towards pathogens and their effect on crop yield and protection.

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Chapter 9

ENDOPHYTIC ASSOCIATIONS OF *AZOARCUS* SPP.

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1. INTRODUCTION

In agriculture, fixed-nitrogen is one of the most widely used fertilizers and the amount used globally continues to increase. In natural ecosystems, the availability of fixed-nitrogen often limits plant growth. It also impacts productivity and affects both the species composition of plant communities and ecosystem processes on all levels. The biological reaction counterbalancing the loss of fixed-N from either soils or ecosystems is biological nitrogen fixation, the enzymatic reduction of N_2 to ammonia. This process, unique to bacteria and Archaea, is estimated to contribute globally between 200-300 million tons of fixed-nitrogen per year: estimates for terrestrial systems are 90-130 million tons fixed-N per year (Galloway *et al*., 1995) and, for marine systems, 100-200 million tons fixed-N per year (Karl *et al*., 2002).

One of the best-studied interactions between microbes and eukaryotes, in which the eukaryotic partner directly profits from biological N_2 fixation (BNF), is the root-nodule symbiosis between rhizobia and legumes. However, the most important crops worldwide, wheat, rice and maize, belong to the Poaceae, which do not naturally form these specialized symbiotic structures. However, it has been shown that some grass-related crops, such as certain Brazilian sugarcane cultivars, can derive a substantial part of the plant nitrogen from BNF in natural soils without any inoculation (Boddey, 1995; Lima *et al*., 1987). In most cases, many different diazotrophic bacteria can be isolated from the roots of grasses or cereals (Engelhard *et al*., 2000; Reinhold *et al*., 1986; Reinhold-Hurek *et al*., 1993b; Stoltzfus *et al*., 1997) and this makes it difficult to determine which microbial diazotrophic partner(s) is contributing the fixed-nitrogen.

Among colonization sites, such as the rhizosphere soil, rhizoplane, and root interior, much of the current research on grass-associated diazotrophs focuses on endophytic bacteria (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998b). Endophytic microorganisms multiply and spread inside plants without either

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causing damage to the host plants or conferring an ecological threat to the plant (Quispel, 1992). Although they invade plants, they cannot be regarded as typical pathogens or endosymbionts. Recent results on their contribution of fixed-nitrogen to their grass host (Hurek *et al*., 2002; Sevilla *et al*., 2001) has fostered research on their lifestyle and mechanisms of interaction with grasses, which may contribute to the use of these organisms for a more sustainable-agricultural practice.

2. THE RISE OF INTEREST IN DIAZOTROPHIC ENDOPHYTES

After it was clear in the 1980s that the N_2-fixing bacteria that had been isolated from gramineous plants during the 1950s to 1970s did not fulfill Koch's postulates (Giller and Day, 1985), diazotrophs colonizing the interior of gramineous plant roots or other tissues became a priority target in cultivation efforts. At that time, convincing evidence was provided that sugarcane can obtain a considerable amount of nitrogen from BNF (see Boddey *et al*., 2003 for a review). Independently, several reports on gramineous plants, such as smooth cord grass (*Spartina alterniflora*) and Kallar grass (*Leptochloa fusca*) growing in environments where primary production was probably nitrogen-limited, provided for the first time conclusive evidence that the endorhizosphere (the root interior including the epidermis cortex region; Balandreau and Knowles, 1978) should constitute the habitat of the most important N_2-fixing bacteria in gramineous plants.

First, there were reports of high numbers (up to 10^8 bacteria per g root dry weight) of novel diazotrophs being cultured out from internal root tissue of plants that were not subject to intensive agricultural practice or breeding (see, *e.g*., McClung *et al*., 1983; Reinhold *et al*., 1986). These data were obtained entirely by the isolation, counting, and identification of bacteria from soil, and washed or surface-sterilized roots. They were trustworthy with the appropriate control required to distinguish endorhizosphere from rhizoplane bacteria (van Berkum and Bohlool, 1980) being carried out. The results showed that the number of bacteria released from the root surface was several orders of magnitude lower than the number of bacteria obtained after homogenization of surface-sterilized roots (McClung *et al*., 1983; Reinhold *et al*., 1986). They indicated that there were previously unrecognized, endorhizospheric bacteria that are very well adapted to life in or proximal to living plant tissues in the natural environment of gramineous plants. All these assumptions were confirmed later for Kallar grass and helped to establish the concept of diazotrophic grass endophytes (see, *e.g*., Reinhold-Hurek and Hurek, 1998a; 1998b).

Second, diazotrophs were found to be highly enriched in roots compared to either the root surface or the soil (see, *e.g*., Watanabe *et al*., 1979; McClung *et al*., 1983; Reinhold *et al*., 1986). A close association between diazotrophs and plants is highly advantageous to the plant because of the more direct access to the nitrogen fixed biologically and also highly advantageous for N_2-fixing bacteria because of a ready supply of substrate (see McCully, 2001; Hurek and Reinhold-Hurek in volume 4 of this series). These data implied already what is becoming increasingly clear, that the most important diazotrophic bacteria in gramineous plants are probably too specialized to persist in soil (Boddey, 1995; Döbereiner *et al*., 1988;

Olivares *et al*., 1996; Reinhold-Hurek and Hurek, 1998a; 1998b) and are maintained by the plant or other sources (Hurek *et al*., 1997).

Third, it was also found that the populations of diazotrophic bacteria on the root surface may be entirely different from those in the root interior (Reinhold *et al*., 1986). The most abundant bacteria isolated from the rhizoplane and the endorhizosphere of Kallar grass were not only taxonomically different (Reinhold *et al*., 1987; Reinhold-Hurek *et al*., 1993b), they also showed distinct physiological capabilities which could be related to the microhabitat from where they had been isolated (Hurek *et al*., 1987). This result confirmed the adequacy of surface sterilization as a method to separate microbial populations living in the vicinity of even field-grown gramineous roots. It also suggested that the rhizosphere of gramineous plants is probably highly structured into environments where BNF can be assumed to be a substantial source of available fixed-nitrogen. It is clear that important N_2-fixing bacteria predominantly establish in the root interior, whereas others are found on the root surface or elsewhere.

This conclusion does not necessarily contradict observations, which were made on domesticated cotton, sweet corn, cucumber, or potato grown under standard fertilization regimes (McInroy and Kloepper, 1994; Sturz, 1995; Mahaffee and Kloepper, 1997), that many bacteria have the ability to colonize plant roots unspecifically (Hallmann *et al*., 1997). In these studies, the characterization of the bacteria was only to the genus level, which might have been insufficient to separate bacteria into those with and those without plant-invasive properties (Reinhold-Hurek and Hurek, 1998b). However, it may be also possible that wild plant species are much more selective with respect to the bacterial microflora with which they form stable associations. There is ample evidence that bacteria differ at the species or even strain level in their capabilities to interact with plants (see, *e.g*., Reinhold *et al*., 1985; Dong *et al*., 2003; Reinhold-Hurek *et al.*, 2004), therefore, a certain capability of a particular bacterium cannot be generalized for the genus.

Plant breeding has been very successful in providing high-yielding plant species under standard fertilization regimes. However, the production of high plant yields in situations, where primary production is nitrogen limited, may require a much higher stringency from the plant side for the microflora selected. This would likely result in a much higher refinement of the microbial populations that are established in roots of undomesticated gramineous plants in nitrogen-poor environments.

Recently, the question has been raised as to whether all bacteria isolated from surface-sterilized roots of gramineous plants should be considered endophytic (McCully, 2001). In roots of gramineous plants, all cells outside the vascular tissue die early so that only bacteria residing inside the stele would be truly endophytic. It is clear that a classification of an endorhizosphere isolate as an endophyte requires more data than can be obtained by isolation alone; such information has been provided for many diazotrophic bacteria. However, irrespective of whether a diazotrophic bacterium inside a gramineous root is endophytic or not, there is probably enough substrate readily available from the still living cells in the inner core (McCully, 2001).

3. *AZOARCUS* SPP. AND RELATED GENERA: STRICTLY PLANT-ASSOCIATED VERSUS SOIL BACTERIA

Azoarcus spp. and related genera form a rather diverse group, which contains strictly plant-associated strains that differ considerably from bacteria living in soils and sediments. They are members of the β–subclass of the Proteobacteria in the *Rhodocyclales*, according to phylogenetic analysis of almost complete 16S rDNA sequences, and are mostly nitrogen-fixing bacteria.

3.1. Phylogenetic Relationships among Plant Isolates of Azoarcus *sensu lato* and Azoarcus *sensu stricto*

In their first taxonomic description, several strains, which were later assigned to the genera *Azovibrio, Azospira* (Reinhold-Hurek *et al*., 1993b), and *Azonexus* (Hurek *et al*., 1997b), were included in the genus *Azoarcus sensu lato*. Except for one strain, all these diazotrophic bacteria had been isolated from a similar source, the roots or culms of a pioneer grass (Kallar grass, *Leptochloa fusca* L. (Kunth) grown in one field in the Punjab of Pakistan, or from fungal resting stages from the same field. They have many physiological features in common and are phylogenetically related according to DNA-rRNA-hybridization studies (Reinhold-Hurek *et al*., 1993b). Therefore, in addition to the three bacterial groups deserving the rank of separate species of *Azoarcus* (*sensu stricto*), three groups of bacteria were included in the *Azoarcus sensu lato*, although they were located on the *Azoarcus* rRNA branch at low $T_{m(e)}$ values (Reinhold-Hurek, *et al*., 1993b) or showed low 16S rDNA similarity (Hurek *et al*., 1997b). These bacterial groups harbored only a few physiologically quite similar strains, rendering a detailed taxonomic description difficult. Later, additional strains were isolated from other sites and other grasses, mainly rice (Engelhard *et al*., 2000). This made it possible to carry out better phenotypic descriptions, for example, by exploring nutritional features typical of all members of a group. In addition, the almost complete 16S rDNA sequences allowed a better phylogenetic analysis.

The closer relatedness of *Azoarcus sensu stricto* with the genus *Thauera* in comparison to the other groups of *Azoarcus sensu lato* showed that the original genus *Azoarcus sensu lato* was not monophyletic (see also Figure 1). Therefore, in a reassessment of the taxonomic structure of this genus, the unnamed groups C and D (Reinhold-Hurek *et al*., 1993b) obtained the rank of different genera, namely *Azovibrio* and *Azospira*, respectively (Reinhold-Hurek and Hurek, 2000). *Azoarcus sensu lato* group E (Hurek *et al*., 1997b) forms a new genus as well, *Azonexus* (Reinhold-Hurek and Hurek, 2000). Interestingly, one of these groups of strains got larger recently after the addition of some previously misnamed isolates. Perchlorate-reducing bacteria that were isolated from samples collected from a primary treatment lagoon of swine waste (in the USA) were described as a new genus (*Dechlorosoma suillum*) (Achenbach *et al*., 2001), even though they had almost 100% 16S rDNA sequence identity to *Azospira oryzae*. We thus performed a polyphasic taxonomic analysis and demonstrated that they were indeed members of *A. oryzae* (the bottom four strains in Figure 1) (Tan and Reinhold-Hurek, 2003).

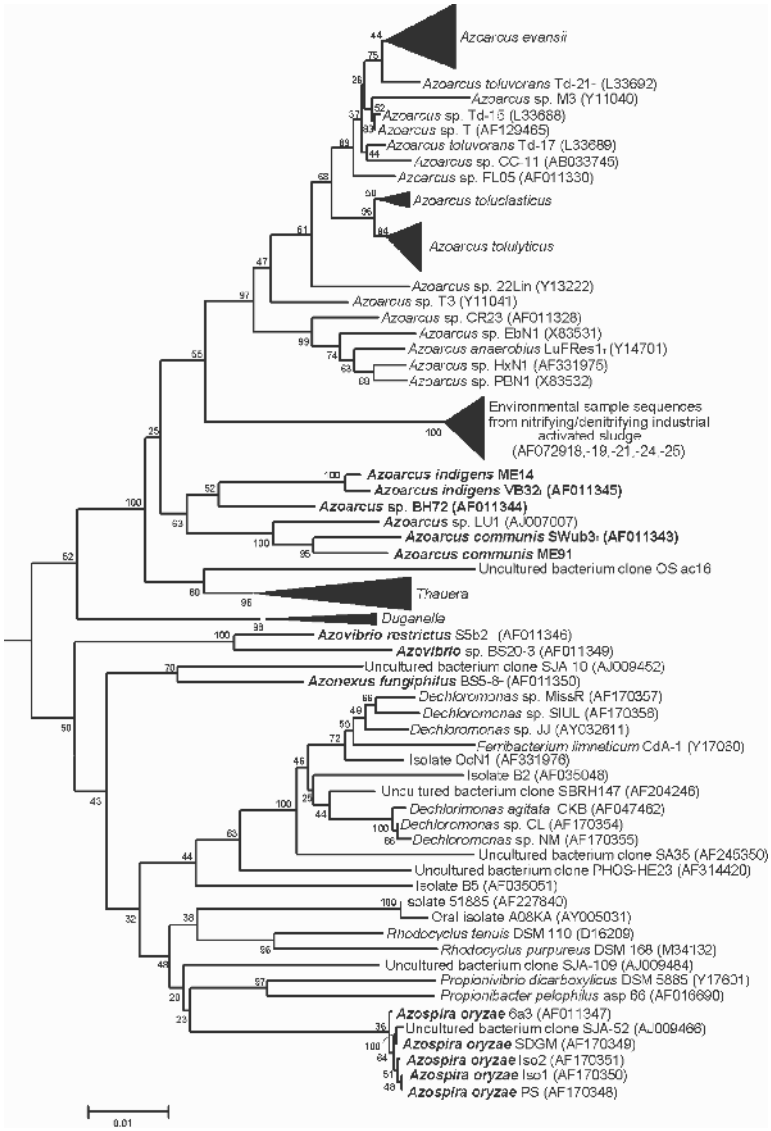

Figure 1. Azoarcus *and related genera: phylogenetic analysis of 16S rDNA sequences (1358 positions). Shown is a subtree of the* Rhodocyclus /Thauera Azoarcus *group derived from an analysis of 158 sequences of ß-subclass Proteobacteria.*
Tree inference was carried out using the neighbour-joining algorithm with a Jukes-Cantor correction with 125 bootstrap repetitions. Clades, which are not of special consideration for this study, are depicted as triangles. Sequence accession numbers are given in parenthesis.

3.2. Differences between Azoarcus *Isolated from Plants and from Environmental Samples*

Azoarcus, the best-studied genus, is phylogenetically most closely related to *Thauera* spp., which are typically soil bacteria (Macy *et al*., 1993; Anders *et al*., 1995; Scholten *et al*., 1999). *Azoarcus* species are located on two different clades (Figure 1), which in part reflects their physiology and ecology. None of the plant-associated species is located on the branch of the soil-borne species that consists of *A. tolulyticus, A. toluclasticus, A. toluvorans, A. buckelii, A. evansii* and *A. anaerobius*; these strains originate mostly from natural or contaminated soil or from sediments. Their occurrence in anoxic sediment or even sewage sludge indicates that their life style *in situ* might be anaerobic. Typical enrichment procedures used strictly anaerobic conditions, with nitrate instead of O_2 as the terminal electron acceptor. In contrast, the plant-associated species, which are also capable of denitrification (Hurek and Reinhold-Hurek, 1995), were enriched under conditions of nitrogen fixation and this may reflect their life style in their habitat. The plant-associated species are *A. indigens*, *A. communis* and *Azoarcus* sp. strain BH72. Strain BH72 deserves the rank of a separate species according to DNA/DNA-binding studies but remains unnamed because it comprises only one strain (Reinhold-Hurek and Hurek, 2000; Reinhold-Hurek *et al*., 1993b; Reinhold-Hurek *et al*., 2004).

Within the genus *Azoarcus*, phylogenetic distances are up to 6%, whereas *Thauera* is 6-7% distant. Thus, *Azoarcus* spp. represents a rather heterogenous group. Moreover, because the phylogenetic distances within the three clades in the *Azoarcus/Thauera* group are similar, both subgroups of *Azoarcus* might also deserve the rank of different genera in future. However, this will require a rigid polyphasic taxonomic analysis so that the same tests are performed on all strains.

The identification of isolates as members of *Azoarcus* by using classical tests is also rendered difficult by this dilemma. Plant-associated *Azoarcus* species and the related genera, *Azovibrio, Azospira* and *Azonexus*, are more similar to each other in several phenoypic key features than to members of the "soil-borne clade" of *Azoarcus*. This heterogeneity of the genus *Azoarcus* is highlighted by the following examples: (i) plant-associated species do not utilize carbohydrates for growth, whereas glucose, maltose, fructose or sucrose can be utilized by many soil-borne species (Reinhold-Hurek *et al*., 2004); (ii) in contrast to the other species, which are straight or slightly curved rods, cells of *A. buckelii* are coccoid (Mechichi *et al*., 2002); (iii) in contrast to all other species that use either O_2 or nitrate as terminal electron acceptor, *A. anaerobius* is strictly anaerobic (Springer *et al*., 1998); (iv) all plant-associated species are capable of nitrogen fixation; this is also the case for *A. tolulyticus* (Zhou *et al*., 1995), whereas other soil-borne *Azoarcus* species (*A. anaerobius, A. buckelii*) do not fix N_2 (Mechichi *et al*., 2002; Springer *et al*., 1998) or have never been tested for diazotrophy. These differing features may reflect differences in either the ecology of these species or the requirements of their habitats, despite of their phylogentic relations.

Some differentiating features of *Azoarcus* and either phylogenetically related or physiologically similar genera are given in Table 1.

Table 1. Differential characteristics of the genera *Azoarcus, Azovibrio, Azospira, Azonexus* and morphologically similar diazotrophs or related bacteria of the *Proteobacteria*

Character	*Azoarcus*	*Azovibrio*	*Azospira*	*Azonexus*	*Herbaspirillum*	*Burkholderia vietnamiensis*	*Azospirillum*	*Gluconacetobacter diazotrophicus*	*Thauera*
Subclass	β	β	β	β	β	β	α	α	β
Cells curved	+	+	+	+	+	-	-	-	-
Cell width (µm)	0.4 – 1.0[a]	0.6 – 0.8	0.4 – 0.6	0.6 – 0.8	0.6 – 0.7	0.3 – 0.8	0.8 – 1.4	0.7 – 0.9	0.7-1.0
Colony colour	Yellowish to beige	Beige	Pinkish translucent	Ochreous	Cream	Cream	Pinkish opaque	Brown	White-yellowish[b]
Fermentative	-	-	-	-	-	-	D	+	-[c]
Growth on sugars	D[d]	-	-	-	+	+	+	+	-[e]
Requirement for cobalamine	-	-	-	+	-	-	-	-	-
Growth on:									
n-Butylamine	+[f]	-	-	-	D	-	ND	ND	ND
4-Hydroxybenzoate	+[g]	-	-	-	+	+	ND	ND	+
Phenylacetate	+[h]	-	-	-	-	+	-	ND	+
Glutarate	+[i]	-	-	-	-	+	ND	ND	+[j]
2-Oxoglutarate	+[k]	-	+	+	+	d	D	ND	ND
n-Caproate	D	-	+	-	-	+	ND	ND	D
Proprionate	D	+	+	-	+	+	+	ND	+[c]
D-Mannose	-	-	-	-	+	+	D	ND	ND
L-Proline	D	-	-	+	+	+	+	+	+[c]

[a] Except for *A. anaerobius* (1.5 µm).
[b] Several strains do not grow on nutrient agar.
[c] Tested for *T. aromatica.*
[d] Positive for soil-borne species, negative for plant-associated species.
[e] Except for *T. selenatis*.
[f] ND for *A. toluvorans, A. toluclasticus, A. anaerobius*.
[g] Negative for *A. evansii*.
[h] Negative for *A. toluclasticus*, d for *A. tolulyticus*.
[i] Negative for *A. indigens*, ND for *A. toluvorans, A. toluclasticus, A. anaerobius*.
[j] ND for *T. mechernichensis*.
[k] Negative for A. tolulyticus, ND for *A. toluvorans, A. toluclasticus, A. anaerobius*.

4. HABITATS AND ECOPHYSIOLOGY

Both plant-associated and environmental *Azoarcus* spp. are found only in terrestrial and not in marine ecosystems, however, both groups of *Azoarcus* spp. strongly differ in their habitats and ecology. All environmental isolates localized in the clade of *A. tolulyticus, A. toluvorans, A. toluclasticus, A. buckelii, A. evansii* or *A. anaerobius* do not originate from living plants, but mostly from soil and sediments. Some of them do not even fix N_2 which was previously thought to be a characteristic feature of the genus *Azoarcus*. Strains belonging to these validly described species have been isolated from non-polluted soils (Song *et al*., 1999) or contaminated soils, *e.g.*, either with unknown contaminations in industrial areas (Song *et al*., 1999) or with petroleum contaminations (Fries *et al*., 1994; Zhou *et al*., 1995). Many strains were also cultured from sediments of creeks (uncontaminated or contaminated) (Anders *et al*., 1995), aquifers (M. Fries *et al*., 1994; Fries *et al*., 1997; Zhou *et al*., 1995), or even from activated sewage sludge (Springer *et al*., 1998). Soil-borne *Azoarcus* spp. are also widespread with respect to their geographical distribution. They have been found, for example, in North America (USA and Canada), South America (Puerto Rico and Brazil), and Europe (Germany and Switzerland).

Enrichment is routinely done under strictly anaerobic conditions, with nitrate as terminal electron acceptor and a variety of aromatic hydrocarbons as carbon sources. In contrast to plant-associated species (Hurek and Reinhold-Hurek, 1995; Reinhold-Hurek *et al*., 1993b), most soil-borne species grow under denitrifying conditions on aromatic compounds, such as toluene or phenol (Song *et al*., 1999; Zhou *et al*., 1995), benzoate (Anders *et al*., 1995), or resorcinol (Springer *et al*., 1998). Due to the anaerobic degradation of aromatic compounds, this bacterial group has received particular attention for biodegradation and biotransformation. Growing numbers of both strains and species are being isolated by many research groups worldwide, in contrast to plant-associated strains (see below). Surprisingly, in contrast to the plant-associated species, none of which has been cultivated, there are numerous studies demonstrating isolation of *Azoarcus* spp. from soils or sediments. This clearly indicates that both bacterial groups occupy entirely different microhabitats. However, the ability to utilize aromatic compounds, which is typical for soil-borne *Azoarcus* spp., is also present to some extent in plant-associated species, which have the ability to grow on aromatic organic acids, like various derivatives of benzoate (Reinhold-Hurek and Hurek, 2000; Reinhold-Hurek *et al*., 1993b). It is tempting to speculate that successful colonization of the grass interior may be facilitated by a capacity to cope with plant aromatic compounds, for example, the phenolic substances involved in lignification.

Members of *Azoarcus* spp., including strain BH72, *A. indigens, A. communis, Azovibrio restrictus, Azospira oryzae* or *Azonexus fungiphilus*, occur inside roots, on the root surface of grasses or in association with fungal resting stages, and have never been isolated so far from root-free soil (Reinhold-Hurek and Hurek, 1998b), with the single exception of a member of *A. communis*, which originated not from plants but from French refinery oily sludge (Laguerre *et al*., 1987; Reinhold-Hurek *et al*., 1993b). Except for this strain, all isolates of *Azoarcus sensu lato* were

originally isolated from Kallar grass (Reinhold *et al*., 1986; Reinhold-Hurek *et al*., 1993b), *Leptochloa fusca* (L.) Kunth, which is a flood-tolerant salt-marsh grass grown since the 1970s as a pioneer plant on salt-affected flooded low-fertility soils in the Punjab of Pakistan (Sandhu and Malik, 1975).

Table 2. Occurrence of plant-associated Azoarcus *spp. and related genera*[a]

Species	Source	Country
Azoarcus indigens	Surface-sterilized culms of Kallar grass	Pakistan
	Endorhizosphere of Kallar grass	Pakistan
	Endorhizosphere of *Oryza sativa*	Nepal
	Fungal sclerotium from rice field	Pakistan
Azoarcus communis	Root surface of Kallar grass	Pakistan
	Black ascocarps from Kallar grass field	Pakistan
	Refinery oily sludge	France
Azoarcus sp. BH72	Endorhizosphere of Kallar grass	Pakistan
Azovibrio restrictus	Endorhizosphere of Kallar grass	Pakistan
	Endrhizosphere of *Oryza sativa*	Nepal
	Fungal sclerotium from rice field	Pakistan
Azospira oryza	Endorhizosphere of Kallar grass	Pakistan
	Endorhizosphere of *O. sativa, O. officinalis*	Nepal
	Endorhizosphere of *O. minuta*	Philippines
	Root pieces of *O. sativa*	Italy
	Fungal sclerotium from rice field	Pakistan
Azonexus fungiphilus	Fungal sclerotium from rice field	Pakistan

[a] *Data from Reinhold* et al.*, 1986; Reinhold-Hurek* et al., *1993; Hurek and Reinhold-Hurek, 1995; Hurek* et al., *1997; Engelhard* et al.*, 2000; Reinhold-Hurek and Hurek, 2000.*

Unlike *Azospirillum* spp., *Gluconacetobacter diazotrophicus*, or *Herbaspirillum* spp., which are widely distributed in the world, *Azoarcus sensu lato* strains had been isolated by only one research group from the same location, indicating that they might be endemic, being perhaps highly adapted either to the saline-sodic soil or to its host plant Kallar grass. There is an ongoing discussion in microbial ecology as to whether bacteria can be endemic at all, or whether "everything is everywhere" (Fuerst and Hugenholtz, 2000). Studies on the distribution of *Pseudomonas* strains have indicated that they might be endemic (Cho and Tiedje, 2000). Another example for putatively endemic bacteria is *Azospirillum halopraeferens*, which originated from the root surface of Kallar grass (Reinhold *et al*., 1986; 1987). Members of this species have never been isolated from any other site although *Azospirillum* spp. are usually easy to culture.

Various other observations suggested that the habitats of *Azoarcus* might be more diverse. These included: (i) detection of *nifH* fragments related to *Azoarcus nifH* sequences (Hurek *et al*., 1997a) in DNA extracts of roots of field-grown rice (*Oryza sativa*) in Japan (Ueda *et al*., 1995); (ii) detection of identical 16S rDNA-fragments related to *Azoarcus* in DNA extracts of Kallar grass roots (Hurek and Reinhold-Hurek, 1995) and spores of a zygomycete colonizing these roots (Hurek and Reinhold-Hurek, 1998); and (iii) isolation of a member of *A. communis* from fungal resting stages occurring in Kallar grass soil (Hurek and Reinhold-Hurek, 1995).

A screening for culturable diazotrophs showed a much wider distribution of plant-associated *Azoarcus* and the related species *A. restrictus* and *A. oryzae*, however, they were mostly from plants grown under flooded, and not under relatively dry, conditions. They were isolated from surface-sterilized roots of several modern cultivars and land races of *Oryza sativa* and wild rice species from the Philippines, Nepal and Italy, as well as from fungal resting stages (see Table 2) (Engelhard *et al*., 2000; Hurek *et al*., 1997b; Reinhold-Hurek and Hurek, 2000). In many cases, especially for *A. oryzae* in wild rice, they were the predominant diazotrophs culturable from the endorhizosphere (Engelhard *et al*., 2000). *Azoarcus*-related *nifH*-gene fragments have also been detected in DNA extracts of soil under *Acacia tortilis* in North Senegal (M. D. Diallo, Ph.D. thesis, University Ghent, 2003).

Nevertheless, it is surprising that *Azoarcus* spp. or related genera, validated by 16S rDNA-sequence analysis, have almost never been isolated from plants by other research groups, except for a survey of Stolzfuss (Stoltzfus *et al*., 1997) on rice in the Philippines. One explanation could be the tendency of this bacterial group to be more commonly associated with wild plants than with modern agriculturally-used cultivars, as has been suggested by our previous survey (Engelhard et al., 2000; see also Hurek and Reinhold-Hurek in volume 4 of this series). In contrast, the majority of studies on diazotrophs is carried out with agricultural crops. We do assume that these bacteria might be more widespread than currently proven, perhaps because they might be more difficult to isolate than other fast-growing diazotrophs. Indeed, these bacteria may be missed during enrichment, when they are overgrown by fast-growing diazotrophs, such as *Azospirillum*, which is facilitated either when the inoculum is not sufficiently diluted or when they are not the predominant culturable diazotroph.

It is important to stress here that *Azoarcus* spp. may occur in an "unculturable" state in nature, where they are highly active but defy attempts at cultivation. It is a common observation in microbial ecological studies that the majority of bacteria present in various ecosystems has not yet been cultivated (Pace, 1997). This has been observed by us for uninoculated and inoculated Kallar grass plants, where *Azoarcus* sp. BH72 could not be isolated, even though its *nifH*-gene mRNA was abundant in roots, indicating high metabolic and nitrogenase activity (Hurek *et al*., 2002). More details on these molecular ecological aspects of diazotrophs and *Azoarcus* spp. is given by us in a chapter in volume 4 of this book series.

5. INTERACTIONS WITH FUNGI

The habitats of plant-associated *Azoarcus* species and *A. restrictus, A. oryzae* and *A. fungiphilus* (Table 2) suggest that they closely interact with eukaryotic hosts. Several of these *Azoarcus*-related genera could be readily isolated from fungal resting stages (sclerotia) that were crushed in enrichment medium (Hurek and Reinhold-Hurek, 1995; Hurek *et al*., 1997b), suggesting they are colonizing surfaces or inner tissues of the fungi. The fungal resting stages were collected from the former Kallar grass field in Pakistan, which afterwards was planted with rice and wheat in rotation. These fungal resting stages were black sclerotia similar to those described in the literature for *Sclerotia rolfsii*, a plant-pathogen deuteromycete that is widespread in subtropical and temperate regions (Puja and Damiani, 1996). A sexual state of this deuteromycete, which resembled the basidiomycete *Athelia rolfsii* (Puja and Damiani, 1996), could be induced. However, there is no complete 18S-rDNA sequence available for either *A. rolfsii* or *Sclerotia rolfsii*. Phylogenetic analysis of 18S rDNA of the sclerotial DNA collected by us suggested that the fungi are indeed *Basidiomycota*, related to the *Ustilagomycetes*. Members of *Ustilago* are plant-associated fungi, which are often phytopathogens. It is thus likely that the *Azoarcus*-harboring fungi also interact with and infect plants.

As in most other plant-microbe systems, at first sight, it might be expected that endomycorrhizal fungi may be involved, especially arbuscular mycorrhizal fungi. For example, the sugarcane endophyte, *G. diazotrophicus*, has been isolated from arbuscular mycorrhizal spores (Boddey, 1995). However, the habitats for *Azoarcus*-infected plants are flooded soils, for both rice and Kallar grass. These anoxic environments are aerated *via* the aerenchymatic tissues of the roots of these flood-tolerant plants, however, they are not sufficiently oxic to allow an efficient colonization with arbuscular mycorrhizal fungi. Thus, Kallar grass roots have been found to be colonized by a variety of other fungi (Hurek and Reinhold-Hurek, 1998).

Because *Azoarcus* and related genera occur on fungal sclerotia in remarkable diversity, these fungal structures might represent an important microhabitat for these bacteria. It might be an inherent feature of these diazotrophs to interact with eukaryotes because the soil-borne members of *Azoarcus* were not isolated from fungal resting stages. In a detailed population study on the same field on which the sclerotia were collected, we were not able to isolate *Azoarcus* spp. or related genera from sclerotium-free sifted soil (Reinhold *et al*., 1986; Reinhold-Hurek *et al*., 1993b). This result indicates that they cannot survive well when free in the soil as has also been proposed for other grass-associated endophytic diazotrophs (Baldani *et al*., 1986).

Initially, it was surprising that root-associated species could be isolated from fungal resting stages present in the soil. However, it is tempting to speculate that fungi, including their resting stages, might serve as niches for survival of diazotrophic endophytes in the absence of plants. They might also be an alternative host and habor a variety of bacterial strains, acting as a “shuttle vector” for new infections of plants. When plant and fungal isolates of the same species, *Azoarcus*

indigens, were compared by fingerprinting methods, some showed almost indistinguishable PCR-profiles (Hurek *et al*., 1997b), supporting this assumption.

The hypothesis that fungal resting stages might act either as intermediate hosts or as vectors for plant infection was tested with sterilized rice seedlings (B. Reinhold-Hurek, unpublished data). Seedlings were grown for 5 days in reagent tubes in plant medium (Egener *et al*., 1999) containing neither N- nor C- sources but agar (4g per L). Then, either a spatula of soil (rice field soil of the former Kallar grass field) or one sclerotium (fungal resting stage) was added, respectively, and plants were grown for a further 4 weeks. Roots were harvested, homogenized, and dilutions were used for semi-solid enrichment cultures in N-free SM-medium as previously described (Reinhold-Hurek *et al*., 1993b). Neither *Azoarcus* nor related genera could be isolated from the soil-inoculated plants, whereas *Pseudomonas* spp. and other uncharacterized strains were obtained. However, *A. oryzae* was isolated from the fungus-incoculated roots as verified by 16S rDNA sequence analysis. Therefore, the fungal sclerotia may host strains of diazotrophic endophytes and transmit them to rice roots. Accordingly, strains of *A. fungiphilus*, which were previously thought to be only fungus-associated, were recently also cultured from rice roots (B. Reinhold-Hurek, unpublished data). The significance of the interactions with fungi is also exemplified in section 6.3. with *Acremonium alternatum* 2003 and diazosome formation.

6. INFECTION OF ROOTS BY ENDOPHYTIC DIAZOTROPHS: AN ACTIVE, SPECIFIC PROCESS?

The assumption that some nitrogen-fixing bacteria may be colonizing the interior of grass roots was mainly derived from circumstantial evidence, *i.e*., through their isolation from surface-sterilized roots (see, *e.g.,* McClung *et al*., 1983; Reinhold *et al*., 1986). Although there were reports that, *e.g.,* using immunofluorescence studies, single cells of *Azospirillum* occur in the cortex of field-grown roots (Schank *et al*., 1979), sound microscopic evidence was lacking on resin-embedded material, which would avoid displacing the bacteria from the root surface during the processes of either sectioning or washing. Their presence was first shown in both immunogold electron microscopic (Bashan and Levanony, 1988) and light microscopic studies (Reinhold and Hurek, 1988), where colonies of either *Azospirillum brasilense* Cd or *Azoarcus* sp. were detected in the cortex of wheat and Kallar grass, respectively. More detailed studies revealed for the first time that diazotrophic endophytes were even capable of invading the stele of grasses (Hurek *et al*., 1991; 1994b), a microhabitat which was previously thought to be invariably sterile in healthy plants. Colonization of aerenchymatic tissues, cortex regions, more rarely stele and xylem of grasses and cereals has meanwhile been demonstrated unequivocally for several diazotrophs, such as *Azoarcus* sp. strain BH72, *Herbaspirillum seropedicae*, *G. diazotrophicus*, and *Klebsiella pneumoniae*, and has been extensively reviewed (James and Olivares, 1998; Reinhold-Hurek and Hurek, 1998a; 1998b).

6.1. Site(s) of Colonization

The cortex region of roots or the aerenchymatic tissue of flood-tolerant plants, such as rice, are the main colonization sites for these diazotrophs (see the reviews listed above). But are these sites really sites of high microbial activity, especially of nitrogen fixation? This is an issue with respect to the function of endophytic diazotrophs. Because aerenchymatic tissue consists mainly of cell walls of lysed cortex cells, this tissue may not look likely at first sight. However, mRNA-based studies may provide good evidence for the localization of nitrogenase activity here, because the transcription of the structural genes, *nifHDK*, is tightly regulated in accordance with conditions favourable for nitrogen fixation (Arcondéguy *et al*., 2001; Dixon, 1998). Moreover, bacterial mRNA is fairly unstable, thus, preventing false positive results due to carry-over effects.

Using *in situ* hybridization with fluorescent probes against the nitrogenase genes of *Azoarcus* sp. strain BH72 on sections of resin-embedded roots, nitrogen fixation was shown to be active in the aerenchyma of soil-grown Kallar grass. The stringency of hybridization suggested that these nitrogen-fixing bacteria were very closely related to *Azoarcus* sp. BH72 (Hurek *et al*., 1997a). Further, reporter gene studies, using either *nifH::gus* or *nifH::gfp* fusions, showed that apoplastic active nitrogen fixation occurs in rice seedlings in gnotobiotic culture with *Azoarcus* sp. (Egener *et al*., 1999). Both infection and expression rates were higher when external carbon sources were added, but they were also detected when concentrations were either negligible (5 mg of malic acid per L) or absent (Egener *et al*., 1999). The question remains open as to which carbon sources are available for the endophytes at these sites. Currently, we are carrying out reporter gene studies using inducible promoters to elucidate this question. A more detailed discussion on how diazotrophic endophytes may gain access to substrates for growth in the apoplast of roots can be found in the chapter by Hurek and Reinhold-Hurek in volume 4 of this series.

6.2. Role of Type IV Pili in Root Colonization

The strain or species differences in endophytic colonization suggest the occurrence of specific mechanisms of recognition, bacterial invasion, and establishment during endophyte-grass-interactions. Is endophytic colonization likely to be an active process involving essential bacterial determinants? Unfortunately, there are only very few data on mechanisms of either invasion by diazotrophic endophytes or establishment of associations involving diazotrophic endophytes. More data have accumulated for *Azospirillum* spp. (Steenhoudt and Vanderleyden, 2000), which are typically rhizoplane colonizers. Which genes are required for establishment of endophytes on or in roots? One of the few known examples of such a requirement is the type IV pili genes of *Azoarcus* sp. strain BH72.

The first crucial event in the infection process of either symbiotic or pathogenic bacteria is attachment to the epithelial cells of the host. Type IV pili play an essential role in mediating bacterial adherence to and colonization of host-cell

surfaces and are virulence factors in many human or animal pathogenic bacteria. They are filamentous cell appendages, which are much thinner than flagella (Figure 2A) and are about 6 nm thick. They are assembled primarily from a single protein subunit, pilin, which consists of approx. 150 amino-acid residues. A short positively-charged leader peptide, which is cleaved off the prepilin, and a highly conserved N-terminal region are characteristic for type IV pilins (Strom and Lory, 1993). The hydrophobic N-terminal region is postulated to form the center of the pilus by hydrophobic interactions of the 54-amino-acid α-helices of the pilin subunits, whereas the *C*-terminal part is exposed to the pilus surface (Forest and Tainer, 1997; Parge *et al*., 1995). This hyper-variable region is the most highly exposed portion (Forest and Tainer, 1997) and the source of strain-specific antigenic variation and interaction with host surfaces (Nassif *et al*., 1993).

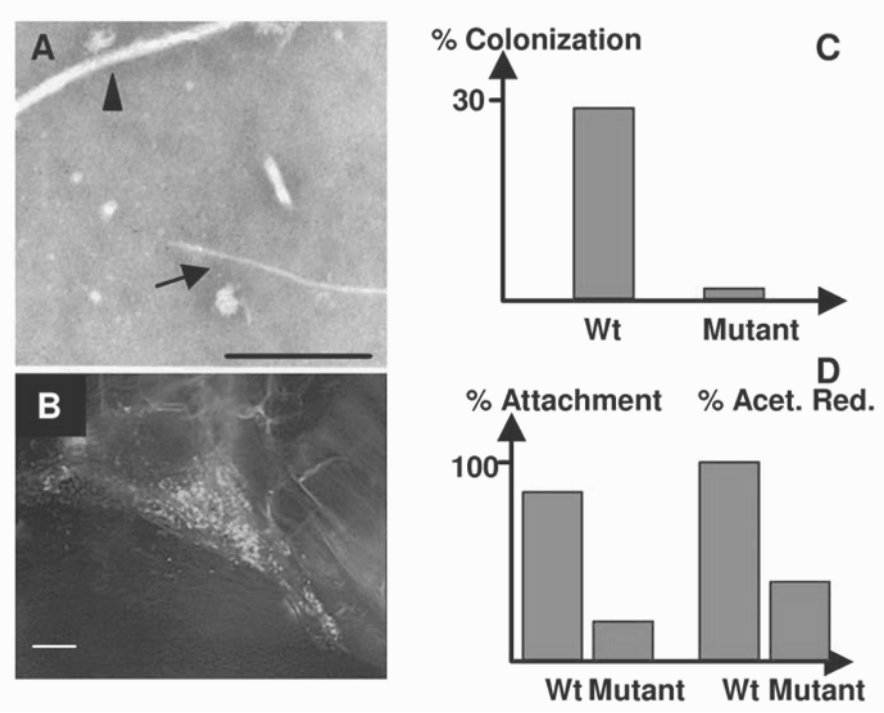

Figure 2. Type IV pili and their role in Azoarcus *sp. strain BH72 root colonization. (A) Transmission electron micrograph showing flagellum (arrowhead) and pilus (arrow); bar, 0.2 µm. (B) Emergence points and lateral roots of a rice seedling in gnotobiotic culture, showing dense colonization (green) with strain BH72; stained with LIVE/DEAD BacLight bacterial viability kit (Molecular Probes); bar, 10 µm. (C) Number of emergence points of lateral roots (as in (B)) colonized by wild type* Azoarcus *or the* pilAB*-mutant, respectively. (D) Co-culture of* Acremonium alternatum *2003 with* Azoarcus *sp. strain BH72 (WT) or* pilAB *mutant. Percentage of bacterial cells attached to the fungal mycelium after two days of incubation, or acetylene reduction per culture during 6 days.*
Figures and data adapted from Dörr et al. *(1998).*

Additional proteins located at the tip of the filament, adhesins, may also interact with host receptors in *Neisseria* (Rudel *et al*., 1995). However, up to now, type IV pili have not been found to be essential virulence factors in plant pathogenic bacteria.

In *Azoarcus* sp. BH72, genes encoding type IV pili were detected and analyzed (Dörr *et al*., 1998). Formation of pili on solid media was dependent on the *pilAB* locus. *pilA* encodes an unusually short (6.4 kDa) putative pilin precursor, which shows 100% homology to the conserved N-terminus of the *Pseudomonas aeruginosa* type IV pilin. *pilB* encodes a 14.2-kDa polypeptide that shows weak similarity to FimF, a component of type I fimbriae of *E. coli*, however up to now, there was no homologue detected in any other bacterium, including sequenced genomes. Because both genes are transcribed in an operon, which is translated and essential for pilus formtion, and because PilB was found to be extruded beyond the cell surface by immunofluorescence studies, PilB is likely to be part of the pilus itself (Dörr *et al*., 1998). This situation is highly unusual because type IV pili usually consist of only one pilin; however, PilB might compensate for the small size of PilA in pilus function.

The emergence points of lateral roots are among the primary sites of colonization of grass roots by *Azoarcus* sp. BH72 (Hurek *et al*., 1994b). Bacterial microcolonies can be easily visualized by using fluorescent dyes, such as the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) (Figure 2B). When gnotobiotic cultures of rice seedlings were inoculated with either wild-type *Azoarcus*, a *pilAB* mutant, or single *pilA* or *pilB* mutants, respectively, the degree of colonization of lateral emergence points was strongly reduced for all mutants (Figure 2C). Complementation with *pilAB* partially restored the wild-type phenotype (Dörr *et al*., 1998). Thus, type IV pili or the *pilAB* genes are essential for efficient adhesion to, colonization of, and ingress into rice roots. An open question is whether they confer any specificity, or whether binding is an unspecific process as long as type IV pili are produced. In many animal pathogens, pili are often responsible for specific binding to target epithelia. Specificity is currently under study for *Azoarcus*-rice interactions, using mutants expressing heterologous pilins. Studies on the transcriptional regulation of *pilAB* are currently revealing unusual features of strain BH72 in comparison to other Gram-negative symbionts and pathogens (Reinhold-Hurek and Hurek, unpublished data).

6.3. Role of Type IV Pili for Interactions with Fungi and Diazosome Differentiation

Another interesting phenotype of *Azoarcus* pilus mutants is the altered behaviour in co-culture with a fungus. An unusual feature of *Azoarcus* sp. BH72 is the formation of intra-cytoplasmic membrane stacks, called diazosomes (Hurek *et al*., 1995). Cells fixing nitrogen under standard conditions (approx. 2 µM of dissolved O_2) do not harbor these membranes. They become apparent in the course of hyperinduction, a physiological state of augmented activity and efficiency of nitrogen fixation in optimized batch cultures at extremely low O_2 concentrations (Hurek *et al*., 1994a; Hurek *et al*., 1995). They are most likely involved in efficient

nitrogen fixation because the iron protein of nitrogenase is mainly localized at these membranes (Hurek *et al*., 1995). A reproducible induction of these structures occurs in co-cultures of strain BH72 (Hurek *et al*., 1995) with an ascomycete strain 2003 (related to *Acremonium alternatum*), which was isolated from surface-sterilized roots of Kallar grass (Hurek and Reinhold-Hurek, 1998). Protein expression and uridylylation status of central regulatory P_{II}-like proteins suggest that this physiological status is indeed quite different from that of standard N_2-fixing cells (Karg and Reinhold-Hurek, 1996; Martin *et al*., 2000).

Interestingly, type IV pili also affect the interaction of *Azoarcus* sp. with this fungal host. Cells of *pilAB* mutants adhere considerably less to the mycelium (Dörr *et al*., 1998) compared to wild-type cultures, where the mycelium harbors most of the bacterial cells tightly attached to it (Dörr *et al*., 1998; Hurek *et al*., 1995; Karg and Reinhold-Hurek, 1996) (Figure 2D). Attachment to the mycelium was hypothesized to be important for diazosome formation and efficient nitrogen fixation (Hurek and Reinhold-Hurek, 1995) and, in corroboration of this suggestion, the decreased adhesion of the mutant also resulted in decreased nitrogen-fixation activity (Figure 2D) and diazosome formation (Dörr *et al*., 1998).

Thus, type IV pili in *Azoarcus* sp. BH72 appear to be important determinants for the adhesion to eukaryotic hosts. The fact that interactions with both plants and fungi are affected, stresses again the hypothesis that, in nature, both types of eukaryotes may be important hosts for *Azoarcus.*

6.4. Infection Process and Production of Plant Cell Wall-Degrading Enzymes

Other findings indicate that the endophytic colonization of grass roots is a process involving active bacterial strategies. For the ingress of bacteria into the root, plant boundaries have to be overcome; either the middle lamella (mainly pectins), for example, for inter-cellular colonization or secondary cell walls (mainly cellulose and hemicellulose) for intra-cellular ingress. It is tempting to speculate that ingress might be mediated by enzymes that degrade plant cell-wall polymers, as has been shown for many phytopathogens (Herron *et al*., 2000; Toth *et al*., 2003). Plant cell wall-degrading enzymes have been detected in *Azoarcus* sp. strain BH72; two types of cellulolytic enzymes are present (Reinhold-Hurek *et al*., 1993a). An exoglycanase, which has cellobiohydrolase activity and also ß-glucosidase activity on ß-1,4-cellooligosaccharides and a wide spectrum of other substrates including xylosides. The endoglucanase preferably attacks oligosaccharides larger than cellobiose and releases larger oligomers from substrates, such as carboxymethylcellulose, and is thus likely to loosen larger cellulose fibers.

In contrast to most phytopathogens, *Azoarcus* sp. BH72 does not metabolize either the substrates or breakdown products (cellolose, cellobiose, glucose). These bacteria do not grow on cellulose, cellobiose, glucose, or any other carbohydrate (Reinhold-Hurek and Hurek, 2000; Reinhold-Hurek *et al*., 1993a; Reinhold-Hurek *et al*., 1993b). Unlike in pathogens, the enzymes are not efficiently excreted into the culture supernatant, but remain bound to the cell surface (Reinhold-Hurek *et al*., 1993a). This might result in a less aggressive attack of plant cells by an endophyte in comparison to plant pathogens. An isogenic mutant was constructed in which the

gene encoding the endoglucanase was inactivated. Indeed, the mutant showed a strong decrease in intracellular infection of root epidermis cells of rice seedlings, suggesting that endoglucanase activity may be important for infection of rice roots by strain BH72 (Reinhold-Hurek and Hurek, unpublished data).

7. CONCLUDING REMARKS

Azoarcus spp. and the related genera, *Azovibrio, Azospira*, and *Azonexus*, harbor species that are closely associated with grasses as diazotrophic endophytes of roots, and may be more widespread than previously thought. The ingress of bacteria into the root is most likely an active process, involving both type IV pili (for adhesion) and plant cell wall polymer-degrading enzymes. The genome of *Azoarcus* sp. strain BH72 is currently being sequenced, financed by the BMBF (Federal Ministry for Education and Science, Germany) in cooperation with the Department of Genetics of the University Bielefeld, Germany (Hurek and Reinhold-Hurek, 2003). Analysis of the sequence data may reveal many surprises regarding similarities and differences to plant pathogens and symbionts. These data will offer the opportunity to study interactions of grasses and endophytes at the level of functional genomics in the future and, therefore, to learn more about the details of both the mechanisms of interaction and molecular cross-talk between the partners. Studies on both partners of the interaction will be facilitated by the fact that the rice genome has already been sequenced (Goff *et al*., 2002; Yu *et al*., 2002).

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Chapter 10

BIOLOGICAL NITROGEN FIXATION IN SUGARCANE

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1. SHORT STORY OF THE SUGARCANE-CROPPING SYSTEM

Sugarcane, *Saccharum* and related genera, belongs to the grass family known until 2001 as Gramineae (Ellis, 1986; Reeder, 1957). In 2001, the Grass Phylogeny Working Group (GPWG) developed a robust phylogeny of the family based on the evolutionary relationships among grasses as well as the structure of grass genomes. The new name of the family is Poaceae, subfamily Panicoide, based on nuclear markers (Gaut, 2002). Currently, this crop is cultivated in the belt 35°N to 35°S and from sea level to 1000m altitude on a wide diversity of soil types (Malavolta, 1994). It is used mainly for sugar and alcohol production.

Around 327 BC, it was an important crop in the Indian subcontinent (Aranha and Yahn, 1987). The approximate center of New Guinea is considered as the home of the *officinarum* species, with its migration occurring mostly by primitive people carrying the sweetest canes with them as they travelled to India, Burma, Indochina, Indonesia, the Solomon Islands, New Caledonia, and Hawaii (Clements, 1980). It has been cultivated in over 120 countries and among member countries of the Food and Agriculture Organization (FAO) of United Nations. It is cropped on an area of 19,682,410 hectares (FAO, 2002). The major sugarcane producers are based in larger countries, such as Brazil (360,556,000 tons) and India (279,000,000 tons), however, the highest yields at more than 100 t ha^{-1} are obtained in countries like Peru, Egypt, and Senegal, compared to Australia and Brazil at only *ca*. 75 t ha^{-1}.

In Brazil, sugarcane is planted on more than 5.2 million ha, 9% of the land under cropping. In 2001, the country exported 11,528 million tons of sugar. Generally, one third of the cane production is directed to the manufacture of hydrated ethanol (95%) representing 12-14 billion L $year^{-1}$. Industrial processing occurs in more than 300 mills/distilleries, all of which are privately owned, and represents an

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agribusiness responsible for 2.3% of the gross national product. Almost three million cars run on 95% hydrous ethanol and all gasoline sold in Brazil contains 20-24% ethanol as biofuel (Baldani *et al*., 2002). In Brazil, this crop consumes more than 240,000 tons of nitrogen (N) fertilizer per year at a cost of around US$150 million.

The response of the sugarcane crop to nitrogen fertilization is very low or null, although the same does not apply to the ratoon crop (annual cane harvests) (Carnaúba, 1990). The dependence of sugarcane on fixed-nitrogen was tested in 135 NPK experiments carried out all over Brazil on the plant crop and only 19 of the studies showed significant yield increases (Azeredo *et al.*, 1986). The crop accumulates between 100-200 kg N ha^{-1} per season (Orlando Filho *et al.*, 1980; Sampaio *et al.*, 1984). Virtually all of this fixed-nitrogen is removed from the field at harvest because the trash, which represents 25% of the senescent leaves, is almost always burned off before cutting and less than 10% of the fixed-N remains in the field in the form of flag leaves (Oliveira *et al.*, 1994).

These data indicate that continuous cropping of sugarcane should deplete soil N reserves such that cane yields should eventually decline. However, such a decline in either yield or soil N reserves is not normally observed even after many decades, or even centuries, of cane cropping. Observations such as these have led several authors to suggest that sugarcane may benefit significantly from fixed-N inputs from biological nitrogen fixation (BNF) and this suggestion was confirmed by the quantification performed by Urquiaga *et al.* (1992). Studies are now underway to determine the major nitrogen-fixing endophytic bacteria responsible for providing the biological fixed nitrogen.

The elimination of N-fertilizer for the production of biofuel crops, such as sugarcane, represents the key to high energy balances because N-fertilizer is produced by reduction of atmospheric N_2 to NH_4, using natural gas as an energy source. The Brazilian Ethanol Program (Pró-Álcool) is the best example (Baldani *et al*., 2002). Sugarcane has been grown in Brazil for centuries, and even today, this crop never receives very high N-fertilizer applications (~ 60 kg ha^{-1}). This probably explains why very large BNF contributions were observed with Brazilian varieties of sugarcane. In fact, among all non-legumes, the highest BNF contribution involves sugarcane. When grown with irrigation, ample phosphate fertilizer, and molybdenum applied as a foliar spray (500 g ha^{-1}), some sugarcane varieties obtain more than 150 kg N ha^{-1} per year from BNF (Boddey *et al*., 2001). Molybdenum is the key minor element of enzymes linked to both nitrogen assimilation (nitrate reductase) and also the nitrogenase enzyme required for bacterial N_2 fixation.

Cane trash is another source of fixed-N input that benefits the sugarcane crop because it constitutes a large organic matter reserve, so improving soil fertility and reducing soil water losses (Oliveira *et al.*, 1994). Moreover, *Gluconacetobacter diazotrophicus* has been found to colonize the trash in high numbers (Reis *et al.*, 1994). Thus, when not removed by burning, cane trash can benefit the plant through both nitrogen fixation and organic fertilization (Patriquin, 1982; Reis *et al.*, 1994).

2. NITROGEN-FIXING BACTERIA COLONISING SUGARCANE: NEW PHYLOGENETIC DATA, PROPERTIES, AND ENDOPHYTIC STATUS

During the 1950s, two species of diazotrophic bacteria belonging to the *Beijerinckia* genera were found in high numbers in the rhizosphere of sugarcane; these were *Beijerinckia indica* and a new species called *Beijerinckia fluminensis* (Döbereiner and Ruschel, 1958). Other diazotrophs were isolated from sugarcane and included species of *Bacillus*, *Azotobacter*, *Derxia*, *Enterobacter*, *Erwinia* and *Klebsiella* (Graciolli and Ruschel, 1981; Rennie *et al.*, 1982; Patriquin *et al.*, 1983; Kennedy and Tchan, 1992). In the 1970s, a new genus, *Azospirillum*, was described. It survives in soil and is enriched in the rhizosphere of various Gramineae, including maize, rice, forage grasses, sugarcane, and palm trees. *Azospirillum* contains some specific strains that are able to infect the plant and multiply within plant tissues (Schloter *et al.*, 1994). These bacteria, however, occur mainly in soil and colonize the rhizosphere of several plants and this localisation, therefore, implies that the N_2 fixed by them is only partially available to the plant.

2.1. Discovery of Novel Species

At the end of the 1980´s, attempts were made to isolate diazotrophic bacteria from the internal plant tissue of aerial parts of sugarcane that are known to have high sugar concentrations, especially the stems. This new habitat enabled the discovery of new species of bacteria that colonize the plant interior without eliciting any symptoms of disease. In 1988, a new species of the *Acetobacter* genus was found inside sugarcane and was first called *Acetobacter diazotrophicus* (Cavalcante and Döbereiner, 1988; Gillis *et al.*, 1989), and more recently renamed as *Gluconacetobacter diazotrophicus* (Yamada *et al.*, 1997; 1998). This new species does not survive in soil and is transmitted from plant to plant mainly *via* plant cuttings (setts) (Reis *et al.*, 1994). These organisms were called endophytes as they live inside the plant tissue either latently or actively colonizing locally or systemically. In some cases, plant-growth improvement is observed (Frommel *et al.*, 1991; Kado, 1992; Kloepper *et al.*, 1992; Chen *et al.*, 1995; Pleban *et al.*, 1995; Halmann *et al.*, 1997; Sevilla *et al.*, 2001).

More recently, *Herbaspirillum seropedicae* (Baldani *et al.*, 1986) and *H. rubrisubalbicans* (Baldani *et al.*, 1996), two new species of N_2-fixing bacteria, were reclassified as endophytes (Döbereiner *et al.*, 1994; Baldani *et al.*, 1997). *H. seropedicae* has been isolated from rhizosphere soil, washed roots and surface-sterilized roots of maize, sorghum, and rice (Baldani *et al.*, 1986), but not from uncropped soil (Baldani *et al.*, 1992). *Herbaspirillum* was originally thought to be a new species of *Azospirillum* due to its similar growth characteristics in semi-solid N-free media, but further analysis indicated a new genus (Baldani *et al.*, 1986).

2.2. Properties of Gluconacetobacter diazotrophicus

G. diazotrophicus was first isolated from sugarcane and since then, it has also been isolated from *Pennisetum purpureum* (Reis *et al.*, 1994), sweet potato (Paula *et al.*,

1991), coffee (Jimenez-Salgado *et al.*, 1997), pineapple (Tapia-Hernández *et al.*, 2000), a grass called *Eleusine coracana* (Loganathan *et al.*, 1999), and several other plants, such as tea (root), mango (fruit), banana (rhizosphere), and ragi (root, rhizosphere and stem) (Muthukumarasamy *et al.,* 2002). Two new diazotrophic species of the same genus were described, but both *G. johannae* and *G. azotocaptans* were not isolated from sugarcane, only from coffee plants (Fuentes-Ramírez *et al.*, 2001). The survival of these endophytic bacteria outside the host plant, either in soil or in rhizosphere environments, was very low (Baldani *et al.,* 1997). Using natural soil, sterile or not, the inoculated *G. diazotrophicus* could not be recovered after 15 days.

G. diazotrophicus is a small Gram-negative bacterium in the form of aerobic rods that show pellicle formation in N-free semi-solid medium with 10% added sucrose; it also grows in media containing 30% sucrose (Cavalcante and Döbereiner, 1988; Reis *et al.*, 1994). It grows and fixes N_2 at pH values ranging from 2.5 to 7.0 (optimum at pH 5.5). Using several sugars, strong acid production is observed resulting in a final pH of 3 or less and this organism continues to grow and fix nitrogen at this pH level for several days. However, it cannot start growing at this very low pH (2.5 - 3.0) (Stephan *et al.*, 1991). *G. diazotrophicus* seems well adapted to sugarcane tissues as it shows best growth with 10% sucrose and at pH 5.5. Alvarez and Martínez-Drets (1995) have shown that this bacterium is unable to either transport or respire sucrose; it needs an extracellular saccharolytic enzyme, levansucrase, which actually provides the bacterium with glucose for growth and fructose for formation of the oligosaccharide, levan. Glucose metabolism appears to proceed exclusively *via* the hexose monophosphate pathway (HMP) because key enzymes of both the Embden-Meyerhof-Parnas (EMP) and Enter-Doudoroff (ED) pathways could not be detected (Attwood *et al.*, 1991; Alvarez and Martínez-Drets, 1995). Further oxidation of the C source proceeds *via* a complete tricarboxylic acid cycle (Alvarez and Martínez-Drets, 1995), which involves a membrane-bound glucose dehydrogenase quinoenzyme (Galar and Boiardi, 1995; Luma *et al.*, 2000).

This bacterium does not possess a nitrate reductase and is consequently able to fix N_2 in the presence of high levels of NO_3^- (Cavalcante and Döbereiner, 1988; Li and MacRae, 1991). In the presence of 10% sucrose, NH_4 assimilation by these bacteria is decreased by 65% compared with growth in 1% sucrose (Boddey *et al.*, 1991; Reis and Döbereiner, 1998). Nitrogenase activity is only partially inhibited by ammonium (Stephan *et al.*, 1991) and, in the presence of 10% sucrose, the bacterium continues to fix N_2 (Reis and Döbereiner, 1998). O_2 damages the nitrogenase system but it is less severe in the presence of high sucrose (10%), possibly due to osmotic protection, because the rate of O_2 diffusion is less at this sucrose concentration (Reis and Döbereiner**,** 1998). These characteristics enable the bacteria to continue to fix N_2 while the plant assimilates fixed-N directly from the soil. This organism is thought to play an important role in providing fixed-nitrogen to infected plants through biological N_2 fixation (Sevilla *et al.*, 1998).

In addition to BNF, production of phytohormones by *G. diazotrophicus* could also play an important role in the association. This bacterium produces indole-3-acetic acid (IAA) in defined culture medium in concentrations of 0.14-2.42 $\mu g\ mL^{-1}$ (Fuentes-Ramírez *et al.*, 1993). In addition, *G. diazotrophicus* can play a role as

biocontrol agent. Pinõn *et al.* (2002) showed that, in liquid culture, this bacterium produced a bacteriocin that inhibits the growth of *Xanthomonas albilineans*, the causal agent of leaf scald disease in sugarcane. Further, an antagonistic effect was observed by *G. diazotrophicus* against the fungus *Colletotrichum falcatum*, a causal organism of red-rot in sugarcane (Muthukumarasamy *et al.*, 2000).

G. diazotrophicus was found to colonize the roots, stems, leaves (Figure 1), and trash of sugarcane (Reis *et al.*, 1994). The bacteria have been localized in the xylem vessels (James *et al.*, 1994; Sevilla *et al.*, 2001) and in the apoplast space of sugarcane planted in Cuba (Dong *et al.*, 1994). It was also isolated from a pink sugarcane mealy bug (*Saccharococcus sacchari*) (Ashbolt and Inkerman, 1990) and even the heat treatment normally applied to control the xylem pathogen, *Leifsonia xyli* spp. *xyli*, did not affect the survival of "this endophyte" or *G. diazotrophicus* residing within sugarcane (Reis *et al*., 1994; Ortega *et al*., 2001). Using a model system to study the transfer of the N fixed by this bacterium, Cojho *et al.* (1993) mixed this culture with a yeast and observed that more than half of the N_2 fixed by the bacteria could be utilised by the yeast, suggesting that plant hosts can also obtain this amount of fixed-N.

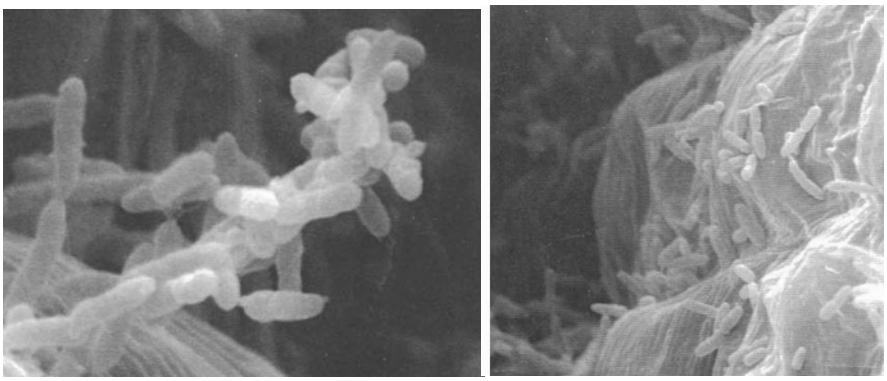

Figure 1. Transmission electron micrographs of G. diazotrophicus *inside sugarcane leaves (cross section) 10 days after inoculation of roots. Courtesy of M. Sevilla.*

The endophytic nature and specificity of this bacterium was confirmed in studies of 12 field-grown Australian sugarcane cultivars (Li and MacRae, 1992). These studies showed that the bacterium was absent or below the detection limit of the method used to quantify the natural population of this species from several other grasses. This result might reflect the limited genetic diversity of this organism (Caballero-Mellado and Martínez-Romero, 1994) and suggest that this organism needs the sugarcane tissue to protect it and to ensure its survival and its passage on to the next crop.

2.3. Properties of Herbaspirillum

Species of *Herbaspirillum* were also found to colonize sugarcane roots, stems and leaves endophytically (Baldani *et al.*, 1996). Until now, this bacterium has been reported in 13 members of the Gramineae, normally colonizing roots (Olivares *et al.*, 1996), but it has also been found in the aerial parts of rice and maize as well as in the stems, but not in leaves, of sugarcane (Olivares *et al.*, 1996). The bacteria are Gram-negative curved rods with polar flagella. They grow best on dicarboxylic acids, gluconate, glucose, and mannitol, and fix N_2 over the pH range of 5.3-8.0 (Baldani *et al.*, 1986; 1992; Ureta *et al.*, 1995). *Herbaspirillum* spp. will grow and fix N_2 under relatively high pO_2 (3%) as compared with *Azospirillum* spp. (2%) (Baldani *et al.*, 1986; Fu and Burris, 1989). *H. seropedicae* expresses nitrate reductase and is able to grow, but not fix N_2, in the presence of fixed-N (yeast extract with NO_3^-), however, nitrogenase activity is only partially inhibited by up to 20mM ammonium (Baldani *et al.*, 1986; Fu and Burris, 1989).

In 1996, *Pseudomonas rubrisubalbicans*, which causes the mottled stripe disease in some sensitive sugarcane varieties, was reclassified as *H. rubrisubalbicans* (Baldani *et al.,* 1996). Along with this reclassification, another group was identified as "species 3" but it included only non-diazotrophic bacteria that were mainly isolated from clinical material, such as wounds and faeces, although a few strains were isolated from sugarcane, sorghum, and maize.

The two diazotrophic species have very similar physiological characteristics; they differ only in the utilization of meso-erythritol (as sole carbon source) by *H. rubrisubalbicans* and of N-acetylglucosamine by *H. seropedicae* and these characteristics can be used to separate them. Also, they differ in optimal growth temperature (30ºC for *H. rubrisubalbicans versus* 34ºC for *H. seropedicae*) and can be distinguished by the use of oligonucleotide probes (Hartmann *et al.*, 1995). *H. seropedicae* has been isolated from sugarcane, sorghum (Hale and Wikie, 1972a; 1972b), rice, and less frequently from palm trees (Baldani *et al.*, 1997) and *Miscanthus* (Kirchhof, *et al.*, 1997), whereas *H. rubrisubalbicans* is isolated from sugarcane and *Miscanthus.*

Microscopy was used to compare a mottled stripe disease-susceptible variety of sugarcane (cv. B-4362 – Barbados) and a resistant one (cv. SP 701143 – Brazil) and large differences were observed. In the susceptible plant, the xylem vessels, intercellular spaces, and sub-stomatal cavities were completely blocked by the growth of *H. rubrisubalbicans.* In contrast, the bacteria were restricted to microcolonies encapsulated within polymeric material in the resistant variety (Olivares *et al.*, 1997). James *et al.*, (1997) inoculated these two species of bacteria into sorghum leaves and observed the same behaviour, the formation of microcolonies in sorghum leaves, for both. A complete review of these two endophytic bacteria has been published by James and Olivares (1998).

2.4. Other Species

Recently, several strains of a new species of *Burkholderia* were isolated from sugarcane plants collected from Pernambuco and South Africa and from maize (*Zea*

mais) and teosinte plants collected in Mexico. It was preliminarily named *Burkholderia tropicalis* (Reis *et al*., unpublished results). Another N_2-fixing bacterium, "*Candidatus* Burkholderia brasilensis" was described by Baldani (1996). These species was isolated from sweet potato (*Ipomea batatas*), sugarcane, rice (*Oriza sativa*), and cassava (*Manihot sculentum*). These two new *Burkholderia* species differ in their use of several carbon sources as well as their colony morphology on a medium containing mannitol at pH 5.5. Genetically, they can be differentiated by the use of primers based on the 16S-rDNA variable sequence (Reis *et al*., unpublished results).

The endophytic occurrence of these diazotrophs may now explain the high contributions that sugarcane can obtain from BNF (Lima *et al.*, 1987; Urquiaga *et al.*, 1992; Boddey *et al.,* 2001). More recent data have shown that the inoculation with a mixture of these endophytes seems to be the best strategy to improve the plant/bacteria association (Oliveira *et al.,* 2000). Similar results were demonstrated for 4 varieties of sugarcane grown in India, where the inoculation with a mixture of different diazotrophic bacteria plus mycorrhiza produced the equivalent of a half dose of the nitrogen recommended for the crop (Muthukumarasamy *et al.,* 1999).

3. CONTRIBUTION OF BNF TO THE SUGARCANE CROP

The first attempt to investigate the possible contribution of BNF to sugarcane was performed by the group at CENA – Centro de Energia Nuclear na Agricultura (Piracicaba, São Paulo, Brazil) in 1975, using ^{15}N-enriched N_2 gas (Ruschel *et al.*, 1975) but utilising only a few plants. Such data do not give information on the agronomic significance of the BNF contribution and, later on, Lima *et al.* (1987) used four sugarcane varieties, planted in large pots containing 64 kg of soil amended with ^{15}N for 22 months (plant crop and one ratoon). The results showed that 40-60% of plant N was derived from plant-associated BNF in the variety CB 47-89. Urquiaga *et al.*, (1992) calculated the contribution of BNF to several varieties, using the ^{15}N-dilution technique, and observed that var. SP 701143 and CB 45-3 were the most promising genotypes reaching a 70% and 68% contribution due to BNF, respectively (as a mean of three years). There are pronounced differences between plant genotypes, showing that the plant plays an important role and that plant breeding will be an important approach for increasing BNF.

Yoneyama *et al.* (1997) made a survey in Brazil, the Philippines, and Mijako (Japan) of the ^{15}N natural abundance of sugarcane plants and neighbouring non-N_2-fixing plants (usually weeds). At many sites, especially in Brazil, the reference plants showed higher ^{15}N abundance than the cane leaves suggesting contributions from BNF. However, it was not possible to compare statistically the ^{15}N abundance of the cane plants and the neighbouring 'reference plants', and usually just two, and sometimes only one, reference plant/s was sampled. Of the 44 cane plants sampled, 32% showed the same or higher ^{15}N abundance than the weeds, and the authors regarded these results as indicating that the cane was obtaining no contribution from BNF at these sites. Also using the ^{15}N-natural abundance technique, 11 sites were studied in several sugarcane plantations in São Paulo, Minas Gerais, and

Pernambuco States of Brazil. In nine of the eleven sites, BNF inputs were significant and ranged from 25% to 60% of the N assimilated (Boddey *et al.*, 2001).

Using a Nif$^-$ mutant of the type strain PAl-5, Sevilla *et al.*, (2001) showed that when fixed-nitrogen was not limiting, growth promotion was observed in plants inoculated with either wild-type or the Nif$^-$ mutant. However, in limiting conditions of fixed-N, plants inoculated with the wild-type generally grew better and had a higher nitrogen content at 60 days after planting. These results indicate that the transfer of fixed-N from *G. diazotrophicus* to sugarcane might be a significant mechanism for plant-growth promotion and these results were confirmed using $^{15}N_2$ gas incorporation. However, it must also be stressed that N_2 fixation by Brazilian sugarcane seems to depend on optimising conditions of water supply, P and K availability, and micronutrients, especially molybdenum (Urquiaga *et al.*, 1992; Boddey *et al*., 2001).

Inoculation with wild type and mutant strains was also performed on rice (Sevilla, unpublished data). The growth of the plantlets increased after inoculation with the wild type, but little increase was observed after inoculation with the Nif$^-$ mutant strain (Figure 2).

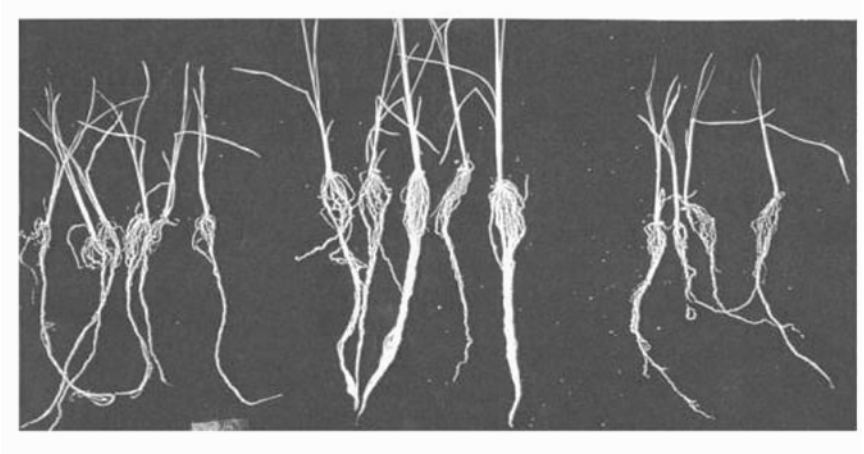

Figure 2. Effect of G. diazotrophicus *on the mass of roots of rice seedlings grown on N-deficient medium. Left, inoculated with the Nif$^-$ mutant MAd3a; Middle, inoculated with wild type strain Pal-5; Right, no inoculation. Courtesy of M. Sevilla (unpublished results).*

4. EFFECT OF N FERTILIZATION ON BNF

Excess fixed-nitrogen can affect the BNF process. In legumes, high levels of soil nitrogen can inhibit nodule formation and cause their early senescence, but the molecular mechanisms involved in these effects are still not well explained. In sugarcane, Vose *et al.* (1981) demonstrated that high levels of mineral-N caused a significant decrease in acetylene-reduction activity (ARA). This effect is probably due to inhibition of nitrogenase synthesis. Fuentes-Ramírez *et al.* (1993) reported

that the association between *G. diazotrophicus* and sugarcane could be severely limited by high N-fertilization rates, which would account for the decrease in ARA. In their study, a much higher number of *G. diazotrophicus* isolates were obtained from crops fertilized with 120 kg N ha^{-1} compared with those fertilized with 300 kg N ha^{-1}. Muthukumarasamy *et al.* (1999a; 1999b) obtained similar results in India for both *G. diazotrophicus* and *Herbaspirillum* spp. These authors suggested that this effect is not a direct negative relationship between the presence of the bacteria and the high levels of mineral-nitrogen present because *G. diazotrophicus* continues to fix N_2 in culture media with high concentrations of NO_3^- (60 mM). It is more probable that the physiological state of the plant is altered by the mineral-nitrogen and this subsequently affects the association with this organism. Rivera *et al.* (1991) also showed, in a field experiment, that high concentrations of mineral-N decreased the population of diazotrophic microorganisms in sugarcane, and that the populations were restored when the mineral-N content of the soil decreased.

dos Reis, Jr. *et al.* (2000) confirmed the results obtained by Fuentes-Ramírez (1993) and Muthukumarasamy (1999a; 1999b) in an experiment in which the response of two sugarcane varieties, planted in a sandy soil and fertilized with 300 kg of N ha^{-1}, was compared with a non-fertilized control. In the sugarcane genotype SP 792312, the N-fertilized plants generally showed higher internal N concentrations and lower numbers of *G. diazotrophicus* compared to the control, whereas the population of *Herbaspirillum* spp. was not affected by N application. This inhibition of *G. diazotrophicus* colonization by N-fertilizer application was not observed for the variety SP 701143. The number of *G. diazotrophicus* present was influenced by the harvest time, decreasing in harvests that coincided with dry periods of the year.

5. GENES FOR NITROGEN FIXATION AND THEIR REGULATION IN *G. DIAZOTROPHICUS* AND *H. SEROPEDICAE*

Field studies of BNF in sugarcane have been complemented by efforts to characterize, at the molecular level, the genetic factors that contribute to the potential for both *G. diazotrophicus* and *H seropedicae* to fix N_2 under various environmental conditions. The results of such studies could lead to development of strategies for genetic manipulation and over-expression of nitrogenase that might result in increased nitrogen fixation, higher production, and release of ammonium to benefit plant growth.

5.1. The nif *cluster of* G. diazotrophicus

The initial characterization of *nif/fix* genes in *G. diazotrophicus* included those encoding the structural genes for the nitrogenase subunits, nitrogenase-enzyme maturation, cofactor biosynthesis, *nif*-gene regulation, and electron-transfer functions. The sequencing of these and flanking regions revealed other genes (Lee *et al.*, 2000; Figure 3).

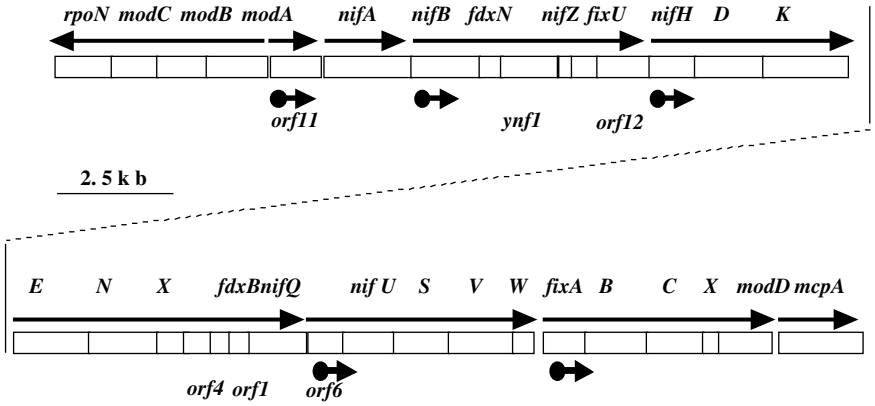

Figure 3. Map of nif *and associated genes in* G. diazotrophicus*.*
Arrows indicate the positions and direction of transcriptional groups.
The ball and arrow symbol indicates the presence of a NifA- and σ^{54}*-dependent promoter.*
Adapted from Lee et al. (2000).

Thirty-two ORF's were identified in the 31-kb sequence obtained. The *nif/fix*-gene cluster characterized here for *G. diazotrophicus* represents the largest single grouping of genes involved in diazotrophic growth or associated aspects of metabolism found in any diazotroph so far studied. The genes include those for nitrogenase structure and function (*nifHDKENX, nifUSVW, nifB, nifQ)*, and for *nif*- and *fix*-gene regulation (*nifA* and *rpoN*, which encode NifA and σ^{N}, respectively, and are required for transcriptional activation of other *nif* genes). The other genes include those responsible for associated functions (*mod* and *fix*) plus a few uncharacterized open reading frames (ORF's). Nearby genes not directly involved in nitrogen fixation include *mcpA*, encoding a methyl-accepting trans-membrane protein that responds to chemotaxis signals, and *modABCD,* encoding a molybdate-transport system. The gene products, FixABCX, probably comprise an electron-transfer chain. Of unknown function with respect to nitrogen fixation but similar to genes in *Rhodobacter capsulatus* are the two ferredoxin-encoding genes, *fdxN* and *fdxB*, and ORF's 1, 4, and 6. The individual gene products are generally most similar to those found in other α–group Proteobacteria, particularly species of the 'rhizobial' genera and *Rhodobacter*. The overall organization of genes in the cluster is most like that of the rhizosphere diazotroph, *Azospirillum brasilense*, and in one region (*nifE* to *nifW*), like that of *R. capsulatus* (which is not known to associate with plants).

G. diazotrophicus and *A. brasilense* are the only two diazotrophs so far characterized that have a *mcpA*-like gene associated with the *nif/fix* cluster. McpA is involved in chemotaxis in many organisms (see Chapter 4 of this volume).

Because both *G. diazotrophicus* and *A. brasilense* are found associated with monocots, the question arises as to whether McpA is required for chemotactic responses to plant exudates. A *mcpA::*kan mutant was constructed and was found to be attracted neither to sucrose nor to a range of other compounds (Lee, unpublished results). Northern analysis of mRNA identified the co-transcription of *nifHDK*, as occurs in most other diazotrophs, and also of *nifENX orf4 orf1 fdxB nifQ* (Lee *et al.,* 2000). This is consistent with σ^N- and NifA-binding sites being upstream of *nifH* and *nifE.* Other probes, *orf6, nifU, fixA*, gave inconclusive results, suggesting either rapid degradation of and/or very little synthesis of mRNA. The transcriptional organization of the other genes was deduced by sequence analysis of promoter regions and/or spacing between adjacent gene start and stop codons.

5.2. Regulation of nifA *Expression*

Expression and function of the *G. diazotrophicus nifA* gene and its product, NifA, which is a transcriptional activator of the other *nif* genes present in all diazotrophs of the taxon (Phylum) Proteobacteria, were also studied (Teixeira *et al.,* 1999). Ammonium repressed expression of *nifA*, but neither σ^N- nor NtrC-binding sites were present upstream of the *nifA* gene. In most other Proteobacteria, ammonium prevents expression of the *nifA* gene by dephosphorylating the NtrC protein, which is the transcriptional activator of *nifA* expression. As a comparison, the expression of *nifA* is not dependent on NtrC and σ^N and is not repressed by ammonium in both *Azotobacter* (Blanco *et al*., 1993) and *Azospirillum,* although NtrC is required for maximal *nifA* expression in this latter genus (Liang *et al*., 1991; 1993). In addition, O_2 had no influence on expression of *nifA* in *G. diazotrophicus* unlike the case for most other Proteobacteria. NifA activity, however, was greatly decreased by either ammonium or O_2, the latter being expected for members of the alpha group of Proteobacteria. The NifA protein in this taxon contains a Cys-X_4-Cys motif, which was shown in *Bradyrhizobium japonicum* to be involved in O_2 inactivation of the NifA protein (Fischer, 1994).

5.3. Reversible Inactivation and Protection of Nitrogenase Activity

In addition to inhibiting the activity of NifA, O_2 also inhibits, sometimes reversibly, the activity of the nitrogenase enzyme in *G. diazotrophicus* and all other diazotrophic Eubacteria and Archaea. There are probably three factors involved in this reversible inhibition of nitrogenase activity in *G. diazotrophicus*. The first two were characterized by Ureta and Nordlund (2002).

The first factor involves a carbon source-dependent respiratory activity to lower intracellular O_2 concentrations during which growth on an efficient respiratory substrate and reductant, such as glucose, results in reversible inactivation of nitrogenase. In contrast, growth on an inefficiently utilized and respired C source, such as pyruvate, did not allow reactivation of nitrogenase activity when O_2 levels were decreased from 20% to 5%.

The second factor involves protection of nitrogenase activity by the FeSII protein (also known as Shethna protein, which was originally identified as being responsible for the "conformational protection" of nitrogenase in *Azotobacter vinelandii* (Scherings *et al*., 1983) under periods of O_2 stress, regardless of C source. The identification of the FeSII protein in *G. diazotrophicus* by antibody detection represents the first time that this protein has been found in bacteria other than species of *Azotobacter* (Ureta and Nordlund, 2002).

The third factor involves colony structure, which also plays a role in protecting nitrogenase from O_2 damage (Dong *et al*., 1994; 2002). Imaging of colonies grown on agar medium by either confocal laser scanning microscopy or light microscopy revealed that at low partial pressure of O_2 (2 kPa), the uppermost bacteria in the colony were very close to the upper surface inside the colony. At high partial pressure of O_2 (20 kPa; atmospheric), the uppermost bacterial cells were located much deeper in the mucilaginous matrix of the colony. Disruption of the colony structure resulted in a decrease in nitrogenase activity. Thus, *G. diazotrophicus* utilizes the distance between the atmosphere and the bacteria to achieve an O_2 gradient that maintains respiration while not inactivating nitrogenase enzyme.

5.4. Role of PII and Paralogues

Further analysis of *nif*-gene regulation in *G. diazotrophicus* has shown that its response to ammonium, the supply of which represses *nif*-gene expression in this and all other diazotrophic Proteobacteria examined, is mediated by one or more proteins that are designated as PII-like and are encoded variously by *glnB, glnK, glnY* and *glnZ* genes in several organisms (see Chapter 3 of this volume). These small (*ca*. 13 kDa) PII proteins are uridylylated at residue Tyr51 under conditions of low fixed-N supply by the GlnD protein, which also removes the UMP moiety in cells grown with high fixed-N supply (*i.e*., growth on ammonium). PII-UMP mediates some responses in Proteobacteria that indicate N-deficiency, such as deadenylylation (activation) of glutamine synthetase and phosphorylation (activation) of NtrC, which is the transcriptional activator of various genes involved in nitrogen metabolism or assimilation.

One *glnB* gene and two *glnK*-like genes (*glnK1* and *glnK2*), all encoding PII-like proteins, were identified in *G. diazotrophicus* (Perlova *et al*., 2003). Mutational analysis of strains carrying null mutations in *glnB* or *glnK1* or *glnK2*, either singly, doubly, or triply, indicated that none of the three PII-encoding genes is required for *nif*-gene expression and, therefore, are not required for NifA activity (in contrast to result obtained for *H. seropedicae*, see below). However, the GlnK2 protein apparently is an inhibitor of *nif*-gene expression in the absence of GlnK1 and GlnB, perhaps because it inhibits the activity of NifA when the other PII proteins are not present In the triple mutant that is devoid of any PII protein, but not in any of the double mutants, nitrogenase expression occurred in the absence or presence of ammonium. The interpretation of these results is somewhat complicated with respect to the various roles of the three PII proteins. More information is needed to elucidate the regulation of nitrogen fixation and the activity of the NifA protein, and possibly other as yet unidentified gene products involved in *nif* gene expression, that

mediate ammonium control (repression) of nitrogenase biosynthesis in *G. diazotrophicus.*

5.5. The nif *Genes and their Regulation in* Herbaspirillum

In contrast to *G. diazotrophicus*, the *nif*-gene cluster, which is so far incompletely identified in *H. seropedicae*, is split within its genome and the distance between the identified clusters, *nifHDKENXorf1orf2orf3* and *nifQmodABCfixXC*, is not known (reviewed in Pedrosa *et al*., 2001; see Chapter 3 of this volume).

In *H. seropedicae*, the other significant nitrogen-fixing bacterial endophyte of sugarcane, expression of *nif* genes is also, as in all other Proteobacteria, regulated by the transcriptional activity of the NifA protein (Pedrosa *et al.,* 2001). As in other Proteobacteria, genes encoding GlnD and PII proteins are also present. Regulation of expression of the *nifA* gene is more complex than in other diazotrophs, with the promoter region having two NtrC-binding elements, three NifA-binding regions, and one integration host factor-recognition sequence upstream of the σ^N promoter recognition sequence.

Sequencing and mutational analyses indicate that the major factor for *nifA* expression is NtrC, positively stimulated by IHF binding (Wassem *et al*. 2000). IHF enhances NtrC activation but inhibits NifA autoactivation of *nifA* expression. The NifA protein is also somewhat unusual in that the N-terminal domain is responsible for inhibiting the transcriptional-activator function of the central and C-terminal domains when ammonium levels are high (and regardless of the N levels in *Escherichia coli*) (Pedrosa *et al.,* 2001). The PII protein, GlnB, appears to be required for NifA function, possibly mediating the inhibitory activity of the N-terminal region. A second PII-like protein is encoded by *glnK* but its function in nitrogen-fixation regulation has not been reported. A more detailed report both on the *nif*-gene organization and their regulation can be found in Chapter 3 of this volume.

6. IS INDOLE ACETIC ACID PRODUCTION AN IMPORTANT FACTOR IN THE ABILITY OF *G. DIAZOTROPHICUS* TO ENHANCE GROWTH OF SUGARCANE?

G. diazotrophicus is apparently beneficial to sugarcane growth by two mechanisms, one of which is dependent, and one not, on nitrogen fixation (Sevilla *et al*., 2001). To determine whether IAA production is a factor leading to sugarcane growth enhancement, genes known to be involved in IAA biosynthesis in other organisms were sought by PCR and complementation strategies. These were not successful. Screening of random Tn*5* mutants of *G. diazotrophicus* strain Pal-5 led to the isolation of strain MAd10, which produced very little IAA. However, the mutation that led to decreased IAA production was not associated with the insertion site of Tn*5* in MAd10 but instead with a gene determined by complementation analysis to be *ccmC* and involved in cytochrome *c* maturation (Lee *et al*., 2004). The *ccm* operon was sequenced and found to encode proteins of *ca*. 50% identity to those of

the *ccm* operon in *Bradyrhizobium japonicum*. Insertion of Kan cassettes into the *G. diazotrophicus ccm* genes cloned on suicide vectors, followed by their reintroduction into the *G. diazotrophicus* genome, led to the construction of several *ccm* mutants. The mutants were IAA^-, producing only *ca*. 5% of wild-type levels of IAA. Therefore, cytochrome *c* is likely to be an essential component of an IAA-biosynthetic enzyme with redox functions in *G. diazotrophicus*. Neither growth rates on several media nor the ability to fix N_2 were influenced in the *ccm* mutant strains; the only phenotype observed was a decrease in IAA production.

The effect of a *ccmC* mutation on plant-growth enhancement was examined, either singly or in combination with a mutation in *nifD*. Regardless of fixed-nitrogen supply, plants inoculated with wild-type Pal-5 were larger than uninoculated plants. Plants inoculated with a *ccmC* mutant or a *nifD* mutant or a *ccmC-nifD* double mutant were no larger than uninoculated plants in conditions of both N-deficiency and N-sufficiency. This result contrasts with that of Sevilla *et al*. (2001), who found that the *nifD* mutant could enhance sugarcane growth under +N conditions. The reason for this discrepancy is not known, but it could be related to the shorter growth period before plants were harvested. These results indicate that both nitrogen fixation and IAA production could be factors that allow *G. diazotrophicus* to benefit plant growth, and that a threshold of input by either factor must be attained before growth enhancement is achieved. Proof of the involvement of IAA in plant-growth enhancement requires having mutant strains with lesions in a gene directly and exclusively involved in IAA biosynthesis.

7. CONCLUDING REMARKS

Field studies, using N balance and ^{15}N-isotope dilution experiments, of sugarcane in Brazil and other countries have consistently indicated that BNF plays a significant role in contributing to the ability of sugarcane to grow in regions where nitrogen-fertilizer application is zero or minimal. The isolation of several nitrogen-fixing bacteria localised in both the rhizosphere and especially inside the plant tissue correlates with these findings.

The sugarcane endophytes *G. diazotrophicus* and *H. seropedicae* have been particularly well-studied. In Brazil, the complete sequencing of both species is under way. Furthermore, the program, called SUCEST (Sugarcane Expressed Sequence Tag) created in 1999, studies functions that include morpho-physiological and genetic aspects of the plant/bacteria association (Baldani *et al*., 2002). Whether either or both of these isolates are the major contributors of biologically fixed nitrogen to sugarcane in agriculture remains a somewhat open question. The application of the tools of molecular genetics to the analysis of nitrogen-fixation and related genes has revealed some unique aspects of the arrangement and regulation of these genes in both organisms, and the use of mutants, which are unable to fix N_2 in microbiologically controlled inoculation experiments of sterile plants, has yielded results that are consistent with and support the hypothesis that *G. diazotrophicus* contributes significant fixed-N to benefit plant growth. Other beneficial plant growth-enhancing factors are probably also significant, such as IAA production by

several plant-associated bacteria, which could stimulate root growth and the efficiency of roots to capture otherwise unavailable N sources in unfertilized soils.

Another possibility that one (or more) of the nitrogen-fixing endophytes inside sugarcane is unculturable and has eluded isolation and identification. This possibility has support from the recent report that many nitrogen-fixing endophytes of Kallar grass cannot be cultured but can be demonostrated by the diversity of *nifH*-gene sequences present among the PCR products obtained using *nifH* primers for amplification of this essential gene for nitrogen fixation (Hurek *et al*., 2002).

The use of single purified strains of, *e.g*., *G. diazotrophicus* in inoculation experiments may not represent what occurs in agricultural situations. It may well be that a consortium of nitrogen-fixing strains will be more significant with respect to the ability to transfer biologically fixed nitrogen to sugarcane than can occur with only one diazotrophic bacterial species (Cojho et al., 1993). In addition, although *G. diazotrophicus* has been reported to be isolated from diverse plant species (tea, coffee, pineapple, banana, *etc*.), these data are still somewhat anecdotal because the studies were neither from several plant replicates in diverse locations nor were N balance/^{15}N studies reported with these host plants. These studies contrast with those for *G. diazotrophicus,* which has been isolated from sugarcane in all the countries in which this crop is grown, and for which the evidence is consistent that its BNF is significant for this host plant.

Even though it is an intriguing possibility that nitrogen-fixing endophytes of sugarcane might be used in the future to extend BNF to other monocots, the related bacterial and plant factors that play an important role both in bacterial colonisation of plant interiors and in effective nitrogen fixation within this environment will need to be identified. One such candidate is the enzyme levansucrase, which is produced only at low constitutive levels in *G. diazotrophicus* when growing *ex planta*. However, after exposure to sugarcane extracts (but not sucrose), it is expressed at levels several-fold higher by induction of the gene-encoding levansucrase, *lsdA* (D. Meletzus, personal communication), which has been previously identified in at least three strains of *G. diazotrophicus* (Arrieta *et al.,* 1996; A. Gardiol, personal communication). Levansucrase is an enzyme secreted by *G. diazotrophicus* that has two functions. First, it hydrolyses sucrose to produce fructose and glucose and, second, it polymerises fructose to produce the exopolysaccharide, called levan. It is hypothesized that levan is the 'glue' that holds microcolonies of *G. diazotrophicus* cells together inside sugarcane and is required for protection of nitrogen-fixing micro-colonies against O_2 damage of nitrogenase.

Results of current and future studies, and the improvement of genetic tools, will lead to a new understanding of signals correlated with plant/bacteria interrelationships. The maintenance of crop yields, based on cycling and processes performed by the beneficial associations, are potential keys for agricultural sustainability.

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Chapter 11

HETEROCYST DIFFERENTIATION AND NITROGEN FIXATION IN CYANOBACTERIA

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1. EARLY HISTORY OF THE ASSOCIATION OF NITROGEN FIXATION WITH HETEROCYSTS

Heterocysts are hard to miss. Whether looking at the fossil record or contemporary cultures or field samples, most filamentous cyanobacteria contain cells that are larger, paler, and more refractile (Figure 1). Historically, as the name given to these cells implies, they have been conjectured to have many functions, some based on careless observations. The first serious proposal that they play a role in nitrogen fixation came from Fay *et al*. (1968), who noticed in the course of a totally different investigation that heterocysts contain an abundance of reducing power, manifest as an ability to reduce a photographic emulsion in the absence of light. This observation coincided more or less with the introduction of a convenient assay for nitrogenase, based on acetylene reduction. Unfortunately, attempts to show that heterocysts contained active nitrogenase foundered on the extreme sensitivity of nitrogenase to inactivation by O_2.

Heterocysts differ from vegetative cells in many ways (Figure 2), some of which will be described below; here, we simply point out that two new layers external to the outer cell membrane, composed of a crystalline glycolipid (Lambein and Wolk, 1973; Gambacorta *et al,* 1995) and a cross-linked polysaccharide (Cardemil and Wolk, 1979), limit the diffusion of gases into the heterocyst and, at the same time, render it resistant to the enzyme lysozyme, which otherwise digests the neuraminic-acid linkages in the outer membrane of the vegetative cells. Figure 3 shows a very high magnification of the region that connects a vegetative cell with a heterocyst, prepared by Kristin Black. The black plug is made of the storage material cyanophycin (see section 3). This material is likely to play an important role in the transport of the products of nitrogen fixation from the heterocyst to the vegetative

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cell. Both the polysaccharide and glycolipid protective layers are essential for nitrogen fixation in air.

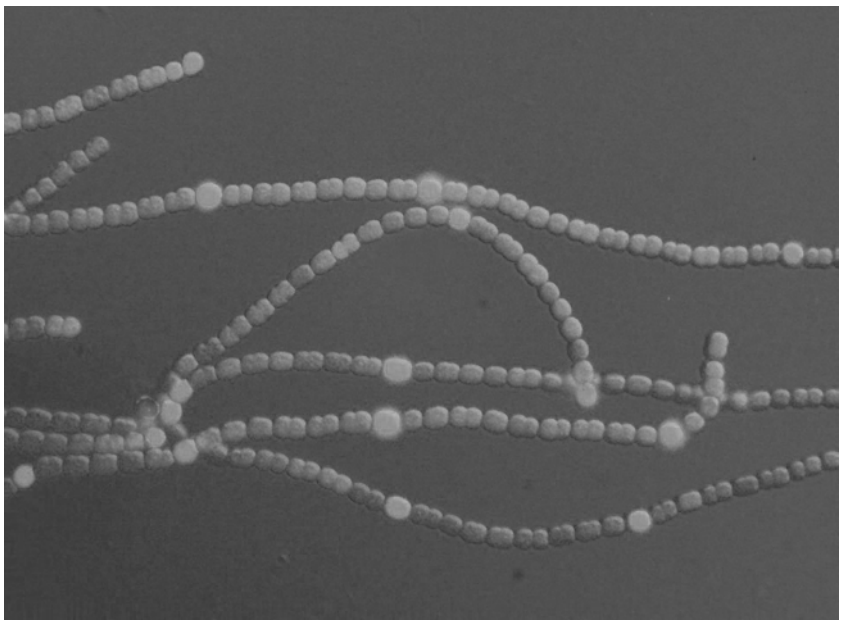

Figure 1. Filaments of Anabaena 7120 at 25 hours after nitrogen stepdown. The bright cells are heterocysts, which are the sites of nitrogen fixation. In this image, they are glowing due to the expression of the Green Fluorescent Protein, whose gene has been fused to the promoter of the hetR gene (see text below).

Eventually, it became possible to isolate active heterocysts, but in the early days, all attempts to do so failed. The first successful demonstration that nitrogenase is located in the heterocysts of *Anabaena* by-passed the activity assay and instead focused on the nitrogenase polypeptides (Fleming and Haselkorn, 1973). The bacterial enzyme had been purified in several laboratories and shown to consist of two components, the so-called MoFe protein, with two kinds of subunits of molecular weight around 56,000 each, and the so-called Fe protein, with subunits of molecular weight around 33,000. Polypeptides of these sizes were found abundantly in heterocysts. Subsequently, it was found that these and other polypeptides were made specifically in heterocysts, whose differentiation could be synchronized by transfer of a culture from ammonia-containing to mineral nitrogen-free medium (Fleming and Haselkorn, 1974).

The protein composition of mature heterocysts reflects the need for the complete conversion of a cell once carrying out oxygenic photosynthesis into a cell that fixes N_2 (Buikema and Haselkorn, 1993; Böhme, 1998). In addition to the enzymes for

synthesis of the glycolipid and polysaccharide wall components, the heterocyst synthesizes all the polypeptides for nitrogen fixation, for electron transport to nitrogenase, for protection from O_2, for nitrogen assimilation and transport to vegetative cells, and for carbon metabolism to provide both reductant and carbon skeletons for nitrogen assimilation. In all, the number of genes differentially expressed in heterocysts could be more than a thousand.

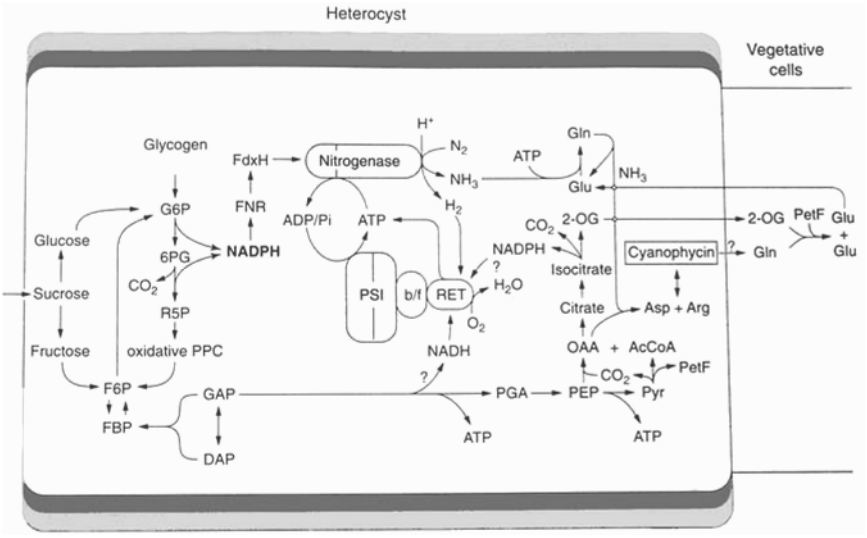

Figure 2. Metabolism in an Anabaena *heterocyst.*
The gray and black layers represent the glycolipid and polysaccharide layers of the heterocyst envelope, respectively. The connection with vegetative cells on either side is not shown at all; refer to the micrograph in Figure 3 for a detailed view.
Energy for nitrogen fixation comes from carbohydrate metabolism, starting with glycogen or sucrose imported from vegetative cells.
Abbreviations: G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate;R5P, ribose-5-phosphate; PPC, pentose phosphate cycle; F6P, fructose-6-phosphate; FBP, fructose bisphosphate; GAP, glyceraldehyde-3-phosphate; DAP, dihydroxyacetone phosphate; FdxH, heterocyst-specific ferredoxin; FNR, ferredoxin nucleotide reductase; PSI, photosystem I; b/f, cytochrome b6/f; RET, respiratory electron transport, including cytochrome oxidase; PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate; Pyr, pyruvate; PetF, vegetative cell ferredoxin (also made in heterocysts); OAA, oxaloacetate; AcCoA, acetyl-CoA; 2-OG, α-ketoglutarate.
Adapted from Böhme, 1998.

The topic of heterocyst differentiation has been reviewed often (Wolk, 1991; Buikema and Haselkorn 1993; Golden and Yoon, 1998). A very useful compilation of facts has been put together by Meeks and Elhai (2002), including a discussion of the control of differentiation by the host plant in symbiotic associations. They also

attempt to model the cell-cell interactions that result in the observed pattern of spaced heterocysts.

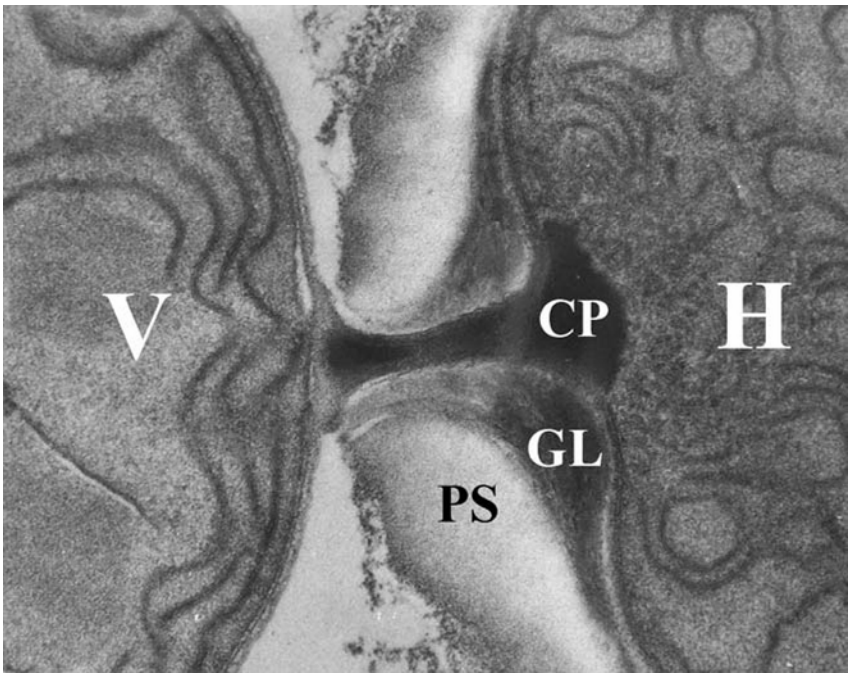

Figure 3. Closeup of the junction between a heterocyst (H) and a vegetative cell (V) of Anabaena *7120 in a fully differentiated culture.*
The polysaccharide layer (PS) and the crystalline glycolipid layer (GL) completely surround the heterocyst, external to the cell wall. The cyanophycin plug (CP) extends from the heterocyst interior to the periplasm or the cytoplasm of the vegetative cell. Transport of the product of nitrogen fixation, glutamine, is thought to involve its conversion to arginine, polymerization into cyanophycin on the heterocyst side, and its depolymerization on the vegetative cell side. Inside the vegetative cell, the arginine can be metabolized to provide glutamine and all the other amino acids.
Electron micrograph (magnification 30,000) courtesy of Dr. Kristin Black.

2. CYANOBACTERIAL NITROGENASE AND *NIF*-GENES ORGANIZATION

The genome sequence of *Anabaena* PCC 7120 reveals one, and only one, set of genes encoding nitrogenase (Mazur *et al*., 1980; Rice *et al*., 1982; Kaneko *et al.,* 2001). These are organized in the same operon structure as in *Klebsiella pneumoniae* and other nitrogen-fixing bacteria (Figure 4), with two exceptions. The *nifD* gene, encoding the alpha subunit of the MoFe protein, is interrupted by an 11-

kb element (Golden *et al.,* 1985) and the fdxN gene is interrupted by a 55-kb element (Golden *et al.,* 1987). Failure to excise these elements from the DNA results in a defective nitrogenase. The elements are normally excised precisely during heterocyst differentiation, using a specific recombinase induced during differentiation in the heterocysts (Lammers *et al.,* 1986; Chastain *et al.,* 1990). This enzyme is encoded in each element itself. Its target sequence is a short sequence directly repeated at the ends of the element. Regulation of the excision appears to be accomplished simply by controlling expression of the recombinase gene. It has been possible to generate strains that have the 11-kb element excised by expressing the recombinase gene (*xisA*) during vegetative growth. Such cured strains have no distinguishing phenotype, so it has not been possible to determine what advantage accrues to the strains that carry the element interrupting the *nifD* gene and that must excise the element in order to survive by fixing N_2.

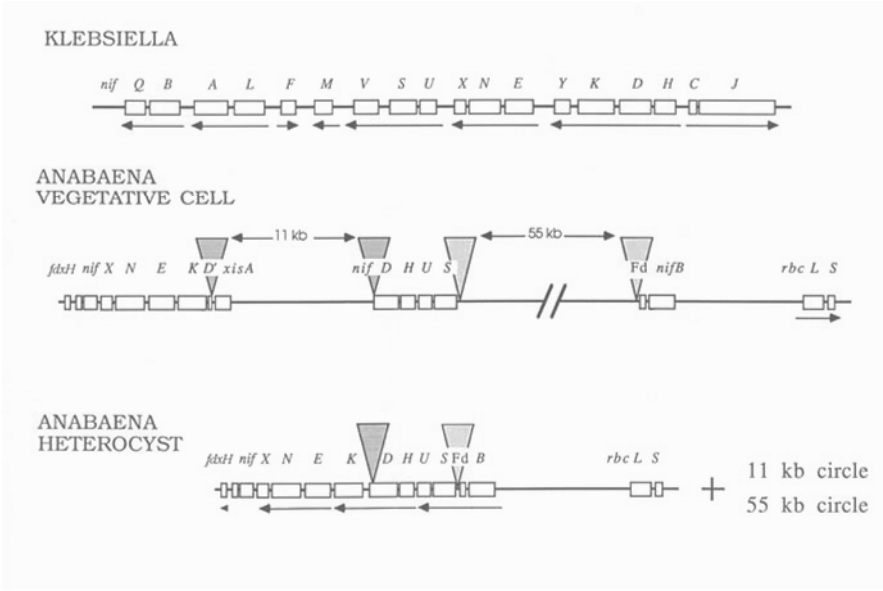
KLEBSIELLA
nif Q B A L F M V S U X N E Y K D H C J
ANABAENA
VEGETATIVE CELL
11 kb
55 kb
fdxH nif X N E K D' xisA
nif D H U S
Fd nifB
rbc L S
ANABAENA
HETEROCYST
fdxH nif X N E K D H U S Fd B
rbc L S
11 kb circle
55 kb circle

Figure 4. Map of the nif *genes of* K. pneumoniae*,* Anabaena *vegetative cells, and* Anabaena *heterocysts.*
Late during heterocyst differentiation, the two intervening elements are excised, the 11-kb element by the product of the xisA *gene, the 55-kb element by the product of the* xisF *gene and two other genes. The* xisA *and* xisF *genes are unrelated to each other and they operate on different directly repeated sequences at the ends of each element.*
Adapted from Haselkorn and Buikema, 1992.

Anabaena PCC 7120 contains the full suite of nitrogen-fixation genes recognized in other diazotrophs, including the ones that are required for assembly of the FeMo-cofactor (reviewed by Böhme, 1998). It contains a single copy of each of the *nif* genes except for *nifH*, which is duplicated. The second copy of *nifH* is

located by itself, not surrounded by any genes that have any connection to nitrogen fixation. There is no alternative system for nitrogen fixation in *Anabaena* PCC 7120, although other strains of *Anabaena* and *Nostoc* do have such systems (Thiel, 1993). In *Anabaena variabilis*, a nitrogenase that functions without Mo, replacing the FeMo-cofactor with a V-containing cofactor, is expressed in vegetative cells under anaerobic conditions (Thiel *et al.*, 1995).

Nitrogen fixation requires a source of ATP, low-potential electrons, and carbon skeletons for assimilation of the fixed nitrogen. Biochemical changes in the developing heterocyst accommodate these requirements (see Figure 2). Quite early in the differentiation process, the enzyme systems that are responsible for fixing carbon (Rubisco and the downstream enzymes) are destroyed by proteolysis. They are replaced by the enzymes of the oxidative pentose pathway, which convert simple sugars to 2-oxoglutarate, *en route* to the formation of glutamate, to which newly-made ammonia is added by glutamine synthetase. The oxidative pentose pathway is fed by glycogen stores initially but, after differentiation is complete and the culture is growing on N_2 as nitrogen source, the carbon for this pathway is supplied by vegetative cells in the form of sucrose. The processing of sucrose produces several moles of reduced NADPH, which feeds electrons to where they are needed, namely, to the nitrogenase reaction and the synthesis of glycolipids and polysaccharides for the cell-envelope components.

The huge requirement for ATP is met in several ways, most of which appear to be essential. One route is by cyclic photophosphorylation through photosystem I, which remains active in heterocysts. The O_2-evolving photosystem II is of course inimical to nitrogen fixation. A second route to ATP is by oxidation of electron carriers, culminating in the cytochrome-oxidase reaction with O_2. A cytochrome oxidase is specifically induced in heterocysts, although it is dispensable (Jones and Haselkorn, 2002). There is a second, constitutive cytochrome oxidase in *Anabaena* PCC 7120 (and in *Anabaena* 29413) that may be able to replace the inducible enzyme when needed (Schmetterer *et al*., 2001; Jones and Haselkorn, 2002; Vallardes *et al.,* 2003).

Electron flow to nitrogenase in *Klebsiella pneumoniae* goes from pyruvate through flavodoxin to the Fe protein. This pathway was not suspected to be present in *Anabaena* because the gene encoding the pyruvate:flavodoxin oxidoreductase, *nifJ*, had not been located. Eventually, the *nifJ* gene was indeed found, but its product was shown to be required for nitrogen fixation only under conditions of iron limitation in the medium (Bauer *et al.*, 1993). Thus, *Anabaena* has two ways to send electrons to nitrogenase. When iron is plentiful, it uses ferredoxin as the electron donor but, when iron is scarce, it uses flavodoxin and the *Anabaena* version of NifJ for that purpose.

The sequence of the NifJ protein in *Anabaena* contains a curiosity. Right in the middle of the gene, there is an insertion of five tandem copies of a seven-base repeat, CCCCAGT. This repeat occurs in many hundreds of copies throughout the *Anabaena* PCC 7120 genome. For NifJ, this insertion results in the (apparent) addition of 12 amino acids, most of which are proline, to the middle of the protein. Unless these amino acids are removed by editing of some kind, they do not diminish the function of NifJ.

3. PATHWAY OF N ASSIMILATION

The pathway of N assimilation in *Anabaena* was determined many years ago (Meeks *et al*., 1977), using radioactive N in the forms of ammonia and N_2 gas. The isotope ^{13}N has a half-life of 20 minutes, so it must be generated (in a cyclotron) and used in a few hours. These experiments showed that the path followed by ammonia, whether fed as such or generated by nitrogen fixation in heterocysts, is addition to glutamate to make glutamine, using the enzyme glutamine synthetase (Thomas *et al*., 1977).

Anabaena has a single gene encoding glutamine synthetase (*glnA*), which is a conventional bacterial enzyme with 12 identical subunits of molecular weight 55,000 (Orr *et al.*, 1981). Expression of the *glnA* gene is enhanced significantly in heterocysts of cultures fixing N_2. The gene has multiple promoters, some of which are nitrogen-regulated (Tumer *et al.,* 1983). The *Anabaena* enzyme is unconventional in the sense that it is not subject to covalent modification by the addition/removal of adenylyl groups in response to the nitrogen status of the cells, as occurs in *Enterobacteriaceae*. There are, however, significant feed-back inhibitors of the enzyme, including certain amino acids (Orr *et al.,* 1981; Orr and Haselkorn, 1982).

No homologues of the *ntrBC* genes are found in cyanobacteria. There is a master regulator of nitrogen metabolism in both unicellular and filamentous cyanobacteria. This protein, NtcA, was discovered in several laboratories (Frías *et al*., 1993; Luque *et al*., 1994;) initially as a binding factor (Bif) that associated with several promoter regions, including that of *glnA, xisA, rbcL*, and *nifH* in *Anabaena* (Wei *et al*., 1993; 1994; Frías *et al*., 1994). A *ntcA*-null mutant was not able to grow with either nitrate or atmospheric N_2 as sole nitrogen source, was unable to form heterocysts, and did not rearrange the *nifD* or *fdxN* elements after induction on a medium lacking combined nitrogen (Wei *et al*., 1994).

NtcA belongs to the same family of the transcriptional regulators as Crp, the CAMP-receptor protein. It functions as both a negative and positive regulator of transcription. Transcription activation by NtcA requires α-ketoglutarate acting directly on the protein, so cyanobacteria monitor the nitrogen status of the cell in the most direct way possible (Muro-Pastor et *al.,* 2001). Cyanobacteria have a *glnB* gene, which encodes a PII protein that trimerizes and can be phosphorylated on serine (Forchhammer and Tandeau de Marsac, 1994; Hanson *et al.,* 1998). Phosphorylated PII is also a positive transcription factor.

The source of glutamate for ammonia assimilation is a problem. There is very little, if any, GOGAT in heterocysts (Martin-Figueroa *et al.,* 2000). So, glutamate must be made from α-ketoglutarate and glutamine *via* the GOGAT reaction in the vegetative cells, which would require that glutamate be transported back into the heterocysts. That seems wasteful. Moreover, α-ketoglutarate is a co-factor for transcription from NtcA-dependent promoters, so it is needed in the heterocysts for that purpose as well. It might make sense to convert α-ketoglutarate to glutamate directly in the heterocyst, using the ammonia from nitrogen fixation, but that is impossible because cyanobacteria do not have glutamate dehydrogenase! At present, the system seems to require that α-ketoglutarate and glutamine be

transported out of the heterocyst and that glutamate be transported back in. There should be a better way!

The fate of the newly-made glutamine is still uncertain. Some of it is probably exported to neighboring vegetative cells. One of the Wolk experiments with radioactive N showed that free heterocysts can export glutamine (dependent upon the addition of glutamate), but the yield of fixed-N exported in these experiments was low. An alternative path for the newly fixed-N that is quite attractive would be to convert the glutamine to arginine and then to polymerize the arginine into the copolymer called cyanophycin. This polymer has a backbone of poly-aspartate, each residue of which is linked to arginine or, in some cases, lysine (Ziegler *et al*., 1998; Berg *et al*., 2000; Hai *et al*., 2002).

Cyanophycin is known to be a storage material for fixed-N, accumulating at the poles of heterocysts in the so-called polar granules. The attraction of this pathway for assimilation is that it would remove soluble amino-acid N from the heterocysts in the form of the polymer, thus relieving repression and feedback on the assimilation processes. The polymer could then be solubilized at the poles of the cell in connection with an active transport of, say, arginine, into the vegetative cells (Richter *et al.,* 1999).

There is some evidence to favor this model. The enzymes that convert glutamine to arginine, as well as the cyanophycin synthetase, increase in activity in heterocysts. Although cyanophycin may be a preferred intermediate in the assimilation of fixed-N, it cannot be essential. Ziegler *et al*. (2001) have inactivated the unique gene for cyanophycin synthetase (*cphA*) in *Anabaena* and have shown that a strain, which is incapable of making cyanophycin, can nevertheless grow decently on N_2 as nitrogen source. However, this growth occurs only at low light intensity; at high light, the knockout strain hardly grows at all. This result suggests that cyanophycin is indeed involved but that, under low light intensity when N_2 fixation is slower, an alternative pathway (*e.g*., direct export of either glutamine or arginine) is sufficient.

The degradation of cyanophycin is accomplished by a single enzyme called cyanophycinase, the product of the *cphB* gene. This 30-kD protein appears to be a serine-type exopeptidase that produces the dipeptide of aspartate and arginine in isopeptide linkage (Richter *et al.,* 1999). Further processing of this dipeptide requires a dipeptidase for which there are candidates in the *Anabaena* genome, but no positive assignment has been made yet. Once again, the group of Lockau has made a trenchant observation. A plant-type aspariginase from *Anabaena*, expressed in *E. coli*, was able to hydrolyze isoaspartyl dipeptides, including the Arg-Asp dipeptide that results from the degradation of cyanophycin by cyanophycinase (Hejazi *et al*., 2002). Thus, *Anabaena* contains all the enzymic machinery needed for the dynamic role proposed for cyanophycin in nitrogen assimilation and storage.

4. CARBON METABOLISM IN HETEROCYSTS

The nitrogenase reaction requires a great deal of ATP and reductant. In the oxygenic photosynthetic *Anabaena*, continued carbon fixation in the heterocyst would be difficult because (i) it would draw ATP and reductant away from

nitrogenase and (ii) photosystem II, which generates O_2, would normally be involved in providing these reactants. This is not to say that nitrogen fixation is impossible in the same cell that fixes carbon. For example, *Trichodesmium* does this and it is quite successful in the oceans of the world (Berman-Frank *et al*., 2001; Orcutt *et al*., 2002; El-Shehawy *et al*., 2003). Nevertheless, in *Anabaena* heterocysts, Rubisco is destroyed, phycocyanin is destroyed, there is little photosystem-II activity, and both nitrogen fixation and assimilation are dependent upon the import of carbohydrate from neighboring vegetative cells once stores of glycogen have been exhausted, following nitrogen step-down.

The nature of the carbohydrate imported from vegetative cells has been studied with varying results. The most likely candidate now appears to be sucrose, synthesized in vegetative cells, transported to, and metabolized in heterocysts by invertase. The relevant experiments were done by Curatti *et al.* (2000; 2002), who studied sucrose-synthase mutants of *Anabaena* 7119, which is almost identical to *Anabaena* 7120. First, they showed that sucrose synthase in *Anabaena* functions in the degradative direction. A knockout mutant of *susA*, the gene encoding sucrose synthase, had ten times more sucrose than the wild type on any medium, and it grew well on N_2. On the other hand, a strain over-expressing *susA*, with ten times more sucrose-synthase activity, had no sucrose and could hardly grow on N_2. Invertase activity was unchanged in these mutant strains compared to the wild type, but it was several-fold higher in cells growing on N_2 than in cells growing on nitrate or ammonia (Vargas *et al*, 2003). All of these results are consistent with the proposal that sucrose carries fixed carbon from vegetative cells to heterocysts.

Invertase working on sucrose yields hexoses, which subsequently are processed through the oxidative pentose pathway to give, ultimately, α-ketoglutarate and reduced pyridine nucleotide. There are many loose ends in this scheme and many untested assumptions. The reduced pyridine nucleotide is thought to contribute electrons to nitrogenase, but the precise route is unclear, and much of that reducing power may be needed for the synthesis of cell-envelope components, such as polysaccharide and glycolipids.

5. GENETIC TOOLS FOR STUDYING CYANOBACTERIAL NITROGEN FIXATION

The modern era of cyanobacterial nitrogen-fixation research began about 20 years ago with the demonstration that plasmid DNA could be transferred by conjugation from *E. coli* to *Anabaena* (Wolk *et al.,* 1984). Various technical problems, such as restriction of incoming DNA, were overcome (Elhai and Wolk, 1988) and the system has been made sufficiently efficient that wild-type cyanobacterial genes can be isolated by complementation of mutants *en masse* (Buikema and Haselkorn, 1991b). Several antibiotic-resistance genes can be used for selection of exconjugants and these include genes coding for neomycin/kanamycin, streptomycin/spectinomycin, erythromycin, and chloramphenicol. DNA can also be introduced into *Anabaena* by electroporation, which has the technical advantage that the first transformed cells which arise do not have to be freed of donor cells by repeated streaking. This advantage is offset by the disadvantage of restriction.

When DNA is transferred by conjugation, it can be methylated in the donor cell to protect it from restriction. Finally, there is a phenomenon, which is poorly documented in the literature but well-known to practitioners; introduction of foreign DNA into *Anabaena* by conjugation is itself mutagenic. Suppose one wishes to determine whether a given cloned gene is required for nitrogen fixation. One way to do so would be to introduce by conjugation a plasmid that contains a portion of this gene, such that homologous recombination with the chromosome results in splitting the gene. If the resulting strain cannot fix N_2, one might conclude that the gene in question plays an essential role in nitrogen fixation. But that may not be the case! That conclusion cannot be drawn until the phenotype has been complemented by the wild-type version of the gene. The reason for this conservatism is that often the very act of conjugation results in chromosome breaks and reorganizations, leading to unexpected mutations elsewhere in the chromosome.

The other potentially confounding factor in cyanobacterial genetics is the tremendous ploidy of the cells. *Anabaena* cells growing in moderate light in complete medium have at least 20 copies of the chromosome. Nevertheless, it is possible to select recessive mutants in a few days (equal to a few generations) after the introduction of a mutagen, indicating that segregation of the chromosomes during division is not likely to be random.

Mutagenesis of *Anabaena* has been done in two principal ways; transposon interruption and chemical mutagenesis. Transposons have the advantage that, in principle, it is possible to recover the interrupted gene by isolating the DNA from the mutant, cutting it, circularizing it, and transforming *E. coli* with it. If the transposon is fitted with both an origin of replication for *E. coli* and a selectable marker for *E. coli*, it should be selectable and one or two sequencing runs will identify the gene that was interrupted. The main caveat here is that it still must be proved that the phenotype of the mutant is due to the particular insertion that was characterized.

Chemical mutagenesis has the advantage that mis-sense mutations can be created. Buikema and Haselkorn (1991b) imagined that some proteins might have multiple functions, some of which are essential while others are conditional. In particular, they envisioned some signaling proteins that participate in both stress responses and in heterocyst differentiation and that, although a deletion of such a protein might be lethal, some point mutations might have a defect only in differentiation. They therefore undertook chemical mutagenesis and used penicillin selection to isolate mutants that could not fix N_2 (Buikema and Haselkorn, 1991b). Most of these had defects in differentiation. They also adapted the conjugation method of Elhai and Wolk (1988) to isolate, by complementation, wild-type DNA fragments that permit these mutants to fix N_2 and to differentiate again (Buikema and Haselkorn, 1991b).

6. REGULATORY GENES REQUIRED FOR HETEROCYST DIFFERENTIATION

Several of the genes, which were recovered by complementation of mutants and required for heterocyst differentiation, are considered here in some detail. The

naming of genes has been a little haphazard. We started with *het*, for those genes that we knew affected heterocyst differentiation more or less completely, such as *hetR* and *hetN*, whereas others that affected the pattern of differentiation were called *pat*, such as *patA*, *patB* and *patS*. Where a gene's function could be assigned more precisely, a more informed name was given. Thus, *ntcA* controls many aspects of nitrogen metabolism, *glnB* is a homologue of the *glnB*-gene encoding PII in *E. coli*, and the *cox* genes are homologues of genes encoding cytochrome oxidase in other bacteria. Table 1 summarizes some of the properties of these gene products.

Table 1. A few of the many genes involved in heterocyst differentiation in Anabaena

Gene product	Function	Expression	Phenotype of gene-disruption mutant	Phenotype of complementation/ over-expression
NtcA	transcription regulator	V and H	Het^-, Fix^-	Not known
GlnB (PII)	transcription regulator	V and H	Het^-, Fix^-	Not known
Het R	autoprotease	H	Het^-, Fix^-	MCH
HetF	not known	H	Het^-, Fix^-	MCH
HetN	regulator, anti-HetR	H	MCH	Het^-
PatA	response regulator	H	terminal heterocysts only	Not known
PatS	peptide, anti-HetR	H	MCH	Het^-
PatB	redox-sensitive transcription	H	Fox^-	bizarre

H, heterocyst; V, vegetative cell; MCH, multiple contiguous heterocysts;
Fox^-, defective in nitrogen fixation in the presence of O_2.

6.1. HetR, an Auto-Protease Positive Regulator of Heterocyst Formation

A *hetR* null or point mutant cannot differentiate (Buikema and Haselkorn, 1991a). It fails to induce many heterocyst-specific genes, including itself. Expression of *hetR* is confined to differentiating cells. Normally, about 10% of the cells differentiate in response to removal of nitrate or ammonia from the medium. If *hetR* transcription is turned on from a regulatable promoter, up to 30% of the cells will differentiate, even in the presence of repressing levels of ammonia (Buikema and Haselkorn, 2001). Purified HetR protein, expressed in *E. coli*, has protease activity (Zhou *et al,* 1998; Dong *et al*., 2000). Its only known substrate is itself, the degradation of which yields a series of peptides. A point mutation (Ser164→Asn)

results in a protein without protease activity and a strain that cannot differentiate. HetR has several other serine residues that can be phosphorylated.

Increased expression of hetR is seen in developing and mature heterocysts in response to fixed-nitrogen limitation. Four likely transcriptional start sites for *hetR* have been mapped and a specific transcript that is positively autoregulated has been identified. Using the copper-responsive *petE* promoter from *Anabaena* sp. PCC 7120, ectopic expression of *hetR* increases heterocyst frequencies and induces heterocyst differentiation under fully repressing conditions (Buikema and Haselkorn, 2001). Co-expression of a reporter gene shows that expression from the *petE* promoter is smoothly induced, depending on the amount of copper supplied. In the heterocyst-pattern mutant, PatA, where terminally positioned heterocysts are formed almost exclusively, expression of the *petE*::*hetR* fusion does not result in the formation of intercalary heterocysts. These results suggest that, although the intracellular concentration of HetR is required for the differentiation decision, PatA plays a role as well.

6.2. PatS, a Diffusible Peptide that Controls Heterocyst Pattern

PatS is essential for the formation and maintenance of a normal heterocyst pattern and is required to inhibit heterocyst formation. Hence, a *pats*-deletion strain can form heterocysts on nitrate-containing medium (Yoon *et al.,* 1998). A *patS-lacZ* translational fusion reveals that PatS levels increase prior to 6 hours after fixed-nitrogen step-down and then return to pre-induction levels by 27 hours (Yoon and Golden, 1998; 2001). The *patS* gene encodes a diffusible peptide. A synthetic pentapeptide, PatS-5 (RGSGR), corresponding to the C-terminal part of PatS, inhibited heterocyst formation at sub-micromolar concentrations. PatS is involved in cell-to-cell signaling and controls heterocyst-pattern formation by lateral inhibition, by diffusing along the filaments and creating a gradient of inhibitory signal (Yoon *et al.,* 1998). PatS appears responsible for the initial patterning of heterocysts upon induction, but HetN (see section 6.3) is necessary for the subsequent maintenance of this pattern (Callahan and Buikema, 2001).

By looking for genes that could bypass heterocyst suppression by PatS, *hetL* was identified. Inactivation of *hetL* did not lead to a phenotype different from wild type, suggesting that HetL may not be essential for the differentiation process (Liu and Golden, 2002). However, the PatS-5 peptide could not suppress heterocyst formation in a HetL over-expression strain. Interestingly, *hetL* is similar to the C-terminal part of *hglK,* a gene described by Blac*k et al*. (1995) required for localization of glycolipids in the heterocyst envelope. HetL consists almost entirely of pentapeptide repeats, as does the C-terminal part of HglK.

6.3. Inhibition of Heterocyst Differentiation by HetN

The gene *hetN* encodes a putative oxidoreductase that suppresses heterocyst formation when expressed from a replicating plasmid in *Anabaena* 7120 (Black and Wolk, 1994). The phenotype resulting from mutation of *hetN*, though, was less

clear, as was the possibility that it was involved in heterocyst differentiation. When Black and Wolk (1994) created insertion mutants of *hetN*, they recovered filaments that displayed any of three phenotypes: MCH (multiple contiguous heterocysts); Het^-; or wild-type. They speculated that second-site mutations might be responsible for these multiple phenotypes, and the true phenotype due to a *hetN* mutation alone remained obscure.

To avoid the complication of second-site mutations that mask a lethal phenotype, the *hetN* promoter in the chromosome was replaced with the copper-inducible *petE* promoter to create strain 7120PN (Callahan and Buikema, 2001). This allowed cells to be grown with copper so that *hetN* was expressed. Subsequent removal of copper from the medium shut off HetN expression, mimicking the $HetN^-$ phenotype. In eight strains carrying the copper-dependent *hetN* gene construct that were tested, a MCH phenotype was observed 48 h after fixed-nitrogen step-down when copper was removed from N^- media. Therefore, as with *patS* (see below), over-expression of *hetN* prevents differentiation, and its absence results in excess differentiation. But *patS* and *hetN* appear to be involved in separate processes because the MCH phenotype of the *P(petE)*::*hetN* strain (7120PN) grown without copper differs from that of a *patS* mutant.

In a *patS* mutant, the MCH phenotype is observed during the initial stage of heterocyst differentiation induced by fixed-nitrogen step-down (Yoon and Golden, 2001). Multiple heterocysts develop simultaneously and, as a consequence, they are all roughly the same size. This is not the case under $HetN^-$ conditions. 24 hours after fixed-nitrogen step-down in Cu-free medium, 7120PN shows a pattern of single heterocysts spaced on average every 10 cells along the filament. It is not until 48 hours post-induction that double and triple heterocysts are observed and, after 72 hours, strings of four and five are seen. The individual heterocysts are of unequal sizes, probably as a result of their different ages. This temporal aspect of the $HetN^-$ phenotype suggests that inhibition by HetN is important after the initial pattern of heterocysts has been established, and maintenance of the pattern becomes paramount as the filament grows by division of the vegetative cells. The later action of HetN is consistent with the Northern blot, which shows transcripts of *hetN* only at 12 hours after fixed-nitrogen step-down in the wild-type (Bauer *et al.,* 1997).

6.4. Relationship of HetN to HetR

In wild-type cells, an increase in *hetR* expression can be seen as early as 30 min after fixed-nitrogen step-down, and expression of *hetR* is both necessary and sufficient to promote heterocyst differentiation (Buikema and Haselkorn, 1993). A *hetR*::*gfp* fusion was introduced into both wild-type *Anabaena* 7120 and strain 7120PN to determine where in the regulatory cascade, with respect to *hetR*, HetN suppresses heterocyst formation (Callahan and Buikema, 2001). When wild-type *Anabaena* 7120 harboring a translational fusion between *hetR* and *gfp* was induced for heterocyst formation, a pattern of proheterocysts fluoresced brightly against a background of lesser vegetative-cell fluorescence after eight hours (see Figure 1). In contrast, strain 7120PN showed no pattern of brightly fluorescent cells in the same medium; all of the cells fluoresced at the low level of wild-type vegetative

cells. So, over-expression of *hetN* does not completely block expression of *hetR*, but it does prevent the patterned, high level of expression found in cells destined to become heterocysts. When expression of *hetN* is prevented by withholding $CuSO_4$ from the medium, groups of cells express *hetR* as indicated by GFP fluorescence starting at *ca*. 20 h post-induction. This pattern of expression is consistent with the MCH phenotype that becomes evident at 48 h. Within the groups of fluorescent cells, often one or more bright cells are flanked by cells of intermediate fluorescence, which is brighter than the majority of cells but less intense than the adjacent cell. This pattern probably reflects the asynchronous expression of *hetR* that is responsible for the delay observed in the MCH phenotype.

HetN also acts downstream of *hetR* transcription to prevent heterocyst development. When *hetR* is over-expressed ectopically, 20-30% of the cells become heterocysts, even when the cells are grown with fixed-nitrogen (Buikema and Haselkorn, 2001). Plasmid pPetHetR contains a P(*petE*)::*hetR* translational fusion (Buikema and Haselkorn, 2001). In strains harboring pPetHetR, *hetR* expression from the plasmid is controlled by copper levels in the same way that expression of *hetN* is controlled in strain 7120PN. In addition to having a MCH phenotype, wild-type filaments containing this plasmid also show decreased spacing between groups of heterocysts. But, when pPetHetR is put into strain 7120PN and *hetN* is over-expressed along with *hetR* from the same type of promoter, only 1% of the cells become heterocysts in fixed-nitrogen-deficient media, and heterocysts are absent in the presence of combined-nitrogen. Therefore, in addition to affecting the pattern of expression of *hetR*, over-expression of *hetN* appears to act downstream of *hetR* transcription to prevent heterocyst development.

Thin layer-chromatography experiments indicate that HetN is not necessary for the synthesis of heterocyst-specific glycolipids. Therefore, HetN does not participate in the elongation of fatty acids for heterocyst glycolipids. The data suggest rather that HetN is involved in the maintenance of heterocyst spacing during growth of the filament after the initial heterocyst pattern has been established. As mentioned above, this interpretation is consistent with Northern analysis of the *hetN* transcript, which shows that *hetN* mRNA is not normally visible until 12 hours after fixed-nitrogen step-down, after the initial pattern has already been established (Bauer *et al*., 1997).

6.5. Relationship of the hetR, patA *and* patS *Genes*

The PAT-1 mutant, which results from a frameshift in the *patA* gene, exhibits an abnormal heterocyst-differentiation pattern (Liang, *et al.,* 1992). Under fixed-nitrogen-limited conditions, it forms only terminal heterocysts in the first 24 hours; filaments typically differentiate a single intermediate heterocyst after 48 hours. This pattern of differentiation is independent of filament length and there is no growth in media lacking fixed-nitrogen.

gfp transcriptional fusions have been used to monitor spatial and temporal expression of *patA* and *patS*. A *patA::gfp* fusion plasmid was introduced into three different *Anabaena* 7120 strains: wild-type; PAT1.1 (the *patA* omega-cassette

insertion mutant); and a *hetR* point mutant, which forms no heterocysts. The cultures were grown in media containing nitrate or ammonia and then transferred to N^- media, and the green fluorescence pattern was recorded. *patA* is expressed at only very low levels in the wild-type strain, consistent with Northern analysis of *patA* RNA levels, where the blots required 16 days of film exposures (Liang *et al.,* 1992). Expression of *patA*::*gfp* was not observed in the *hetR* point mutant. The appearance of fluorescence at 3-4 hours post-induction in the *patA*-mutant strain corresponds to the estimated time for GFP to fold and become fluorescent. This is also consistent with a Northern blot, which shows very early expression of *patA* following fixed-nitrogen step-down (Liang *et al*., 1992). *patA* expression is much stronger in a mutant lacking a functional *patA* gene. This result suggests that PatA suppresses its own expression, either directly or indirectly. The *patA* gene appears to be expressed at equivalent levels in heterocysts and vegetative cells (Figure 5 A).

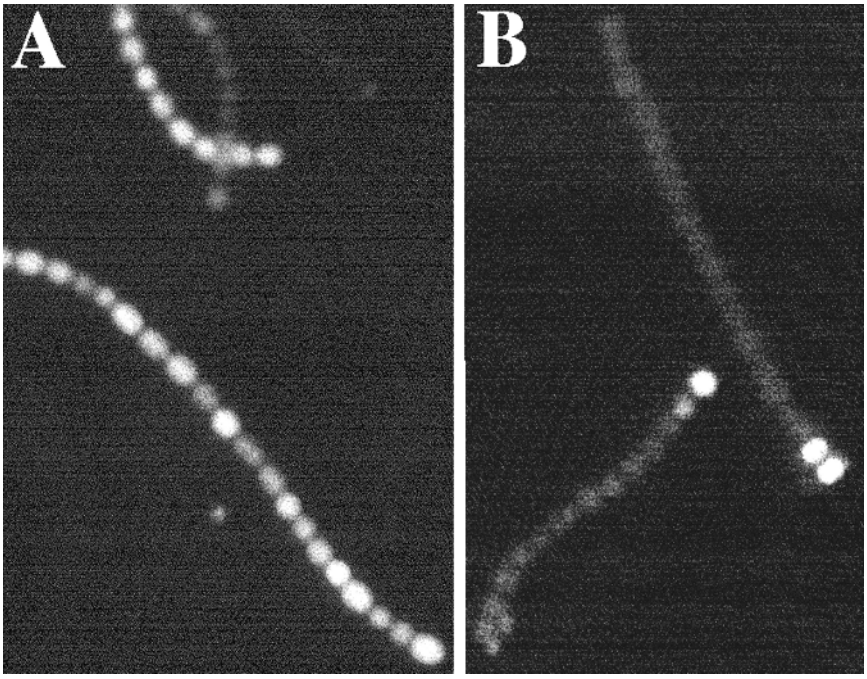

Figure 5. GFP fluorescence from (A) patA::gfp *and (B)* patS::gfp *transcriptional fusions expressed in a* patA*-insertion mutant (PAT-1.1).*
Photo courtesy of Jennifer Moran.

To address the effect of a *patA* mutation on *hetR* expression, two *hetR* fusions were introduced separately into a *patA* mutant. The first, a *hetR::gfp* translational fusion, resulted in fluorescence in only the few terminal heterocysts that differentiated. Therefore, the *patA*-gene product is necessary for *hetR* expression in

intercalary heterocysts. The second construct, a transcriptional fusion between an inducible promoter and *hetR*, failed to bypass regulation by PatA and resulted in a *patA*-mutant phenotype. Thus, PatA acts downstream of *hetR* expression. These seemingly contradictory results can be explained in two ways; either PatA could directly or indirectly interact with HetR to positively control HetR autoregulation or PatA could inactivate an inhibitor of HetR expression.

The carboxyl-terminal third of PatA is similar in sequence both to all of CheY from *E. coli* and to the amino-terminal regions of the response regulators in the two-component regulatory systems of Eubacteria. The four amino-acid residues that are involved in phosphorylation of CheY are conserved in PatA, although phosphorylation of PatA has not been demonstrated yet. The putative phosphorylation site of PatA is in the carboxyl-terminal third of the protein.

We also followed the expression pattern of a *patS*::*gfp* transcriptional fusion in the *patA* omega-cassette insertion mutant (PAT1.1). Yoon and Golden (1998; 2001) have shown that PatS is a diffusible inhibitor of heterocyst differentiation. In the wild-type *Anabaena* strain, *patS*::*gfp* is expressed in the heterocysts and proheterocysts. This is also the case for the PAT1.1 strain, which differentiates mainly terminal heterocysts, with *patS*::*gfp* expressed in these terminal cells (Figure 5B). The level of GFP in the terminal proheterocysts and heterocysts appears to be the same as the level of GFP in the wild-type heterocysts.

We conclude that PatS is produced by developing heterocysts to block the differentiation of adjacent vegetative cells. The *patS* gene is expressed early after differentiation has begun. The *patA* mutant is inhibited from forming non-terminal heterocysts and it expresses *patS* only in the terminal cells. This result implies that PatA is involved in an early step in the differentiation decision of the filament, and that the function of PatS is not so much to generate a *de novo* pattern, but to either control or fine-tune an existing pattern.

The *patS::gfp* fusion is not expressed at all in a HetR-mutant strain. Because *patS* expression requires HetR, it is not surprising that the pattern of *patS*-gene expression follows that of HetR, namely, in proheterocysts and mature heterocysts.

*6.6. Heterocysts of the PAT-2 (*patB*) Mutant Cannot Protect Against O_2*

PAT-2 is a mutant strain of *Anabaena* 7120 that exhibits normal growth under nitrogen-replete conditions, but bleaches and dies 4-6 days after removal of combined nitrogen (Liang *et al.*, 1993). This strain contains a frameshift mutation in the *patB* gene. PAT-2 exhibits a normal initial pattern of heterocyst differentiation, but accumulates multiple contiguous heterocysts after 3-4 days. GFP, expressed under the control of a heterocyst-specific promoter (*hetR*), was used to demonstrate that strongly oxidizing conditions persist in PAT-2 heterocysts long after differentiation has been completed (W. Buikema, unpublished data). In wild-type heterocysts, the GFP fluorescence dims after 2-3 days due to the reducing environment in these cells. In contrast, the PatB mutant heterocysts remain bright after 3 days, suggesting the continued presence of O_2. Unlike other *Anabaena* mutants that have high O_2 levels in their heterocysts due to defects in the formation of the protective envelope, the glycolipid and polysaccharide layers of the cell wall

in PAT-2 appear to be fully formed and intact. These results suggest that, in the PAT-2 mutant, inactivation of the nitrogenase complex by oxidation in the first generation of heterocysts induces subsequent rounds of heterocyst differentiation and ultimately results in death.

The *patB*-gene sequence resembles those of the O_2-sensitive transcriptional regulators of the FNR family. *patB* encodes a protein containing two ferredoxin-like [4Fe-4S] domains at the N-terminus and a helix-turn-helix DNA-binding motif at the C-terminus. The *patB* frameshift mutation in PAT-2 leaves the N-terminal 75% of the ORF intact, but eliminates the DNA-binding motif (Liang *et al*., 1993). Like the original PAT-2 mutant, deletion mutants of the *patB* ORF differentiate multiple contiguous heterocysts after 3 days in nitrogen-depleted medium. Death of Δ*patB* cultures in N^- media is more rapid than that of frameshift mutant cultures, being complete within 4 days. These results are consistent with PatB being responsible for the redox-specific control of multiple genes in the heterocyst.

6.7. patB *is Expressed under Nitrogen-depleted Conditions and Only in Heterocysts*

Expression of *patB* is induced in response to nitrogen starvation, with a 1.65-kb message appearing three hours after fixed-nitrogen step-down and steadily increasing for at least 24 hours (Liang *et al.,* 1993). When a transcriptional fusion of the *patB* upstream elements to *gfp* is expressed in *Anabaena* 7120, a heterocyst-specific expression pattern is observed. Expression is at a low level and is not visible until 16 hours after removal of combined nitrogen. The appearance of GFP fluorescence significantly later than the appearance of the *patB* message may be due to the folding time of GFP and the low level of expression. This expression pattern is preserved in the *patB* frame-shift and deletion mutants, indicating that PatB is not required for its own cell-specific expression.

The putative structure of PatB suggested that its function might be to sense redox conditions through its iron-sulfur domains and to regulate transcription through its helix-turn-helix domain. One potential target of *patB* is a novel *Anabaena* 7120 cytochrome c oxidase operon. Cytochrome c oxidase (type aa3), which is the terminal oxidase in the respiratory electron-transport chain, has a higher activity in heterocysts than in vegetative cells (Schmetterer *et al*., 2001). This high respiratory activity may help maintain the microaerobic environment needed in heterocysts to protect nitrogenase (Peschek *et al*., 1991; Schmetterer *et al*., 2001; Valladres *et al*., 2003). Strain 7120 has two operons that encode full cytochrome oxidases. One, *coxBACI*, is expressed constitutively in vegetative cells and is induced slightly following combined-nitrogen step-down. The other operon, *coxBACII*, is induced at least 25-fold and the transcript is found mostly in heterocysts, based on results with GFP fusions (Jones and Haselkorn, 2002).

6.8. Model of Regulation

Figure 6 summarizes some of our knowledge of the regulatory circuit that leads to heterocyst differentiation. In this model, NtcA (see section 3) appears to control the

early events of differentiation, although it is not specific to the differentiation process (Herrero *et al*., 2001; Meeks and Ehlai, 2002). In contrast, HetN, PatA and HetC play a specific role in early events of the differentiation *per se*. Note that ectopic expression of *hetR* leads to differentiation of heterocysts but not to nitrogen fixation because, among other problems, the *nif* genes are not expressed or rearranged under the special conditions of that experiment. HetC, a protein required for morphological differentiation of heterocysts, shows similarity to ABC transporters (Khudyakov and Wolk, 1997). No mature heterocysts are found within *hetC* filaments; cells with low fluorescence can be seen after 24 hours of nitrogen starvation. A similar pattern is seen in *hetP* mutants; *hetP* is a gene located downstream of hetC (Khudyakov and Wolk, 1997). Both hetR and *hetC* are under the control of NtcA.

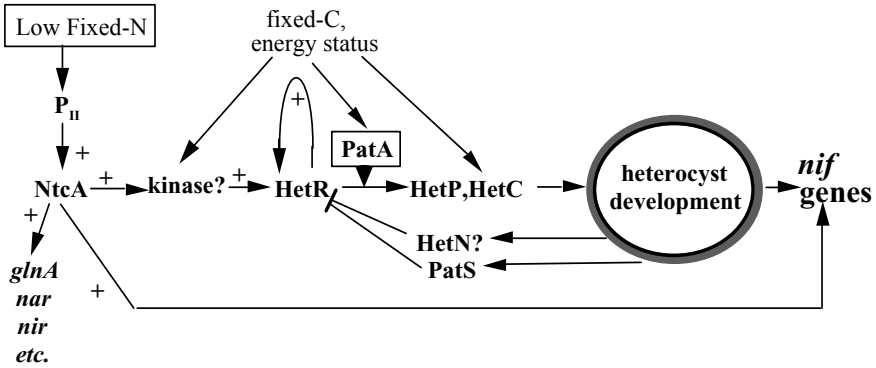

Figure 6. A working model for the molecular interactions among the gene products that govern heterocyst differentiation and pattern formation in Anabaena*.*
In the model, PatA modifies HetR to allow its conversion to its active form, which either directly or indirectly regulates transcription of hetR and other genes necessary for heterocyst differentiation. PatS, the small peptide inhibitor, probably reduces HetR activity or expression. PII is the trimeric regulatory protein (GlnB) that is controlled by addition of UMP in E. coli *but is modified by serine phosphorylation in* Anabaena*. PII responds to the ratio of glutamine to α-ketoglutarate. So does the transcription factor NtcA, which is stimulated by α-ketoglutarate. Numerous protein kinases and response regulators are hidden in the arrows.*

Sigma factors are also likely to be necessary during development. Although many of them have been identified, the requirement of specific sigma factors has not yet been demonstrated (see Brahamsha and Haselkorn, 1992; Böhme, 1998; Meeks and Ehlai, 2002). All of the downstream genes that are required for O_2 protection, such as *patB* and the heterocyst glycolipid and polysaccharide synthesis genes, as well as nitrogenase and the genes for proteins that transfer electrons and protons to nitrogenase, are included in the circle labeled heterocyst differentiation.

6.9. Genome-Wide Expression Analysis in Anabaena

The completion of the genome sequence by the group at Kazusa has made it possible to contemplate global approaches to the regulation of gene expression during heterocyst differentiation. The first results of such experiments are now available, thanks to the *Anabaena* genome project in Japan (Ehira *et al*., 2003). These investigators looked at the transcripts present at zero, one hour, 3 hours, 8 hours and 24 hours after removal of combined nitrogen from a culture of *Anabaena*. They also prepared RNA from a preparation of heterocysts that was about 80% enriched. The results are fascinating. There are at least 19 sets of genes, in terms of the time of maximum transcription, that are up-regulated in response to nitrogen deprivation. We can call these genes early, middle and late, by analogy with the situation in phage infection of bacteria. In all cases, most of the genes have unknown functions.

Among the early genes are *petC, nirABCD, nrtD*, and *narB*. The middle genes include *fdxB, cox BACII, hepC, hepB, devABC*, and *susA*. The late genes, which turn on only after heterocyst differentiation, include all the *nif* genes, the *hup* genes, and the *xis* genes, as well as *coxBAI, hglE, hetM*, and *hetN*. All of the late genes, and some additional ones, are enriched in heterocysts. Some transcripts are depleted in heterocysts, *e.g*., *rbcLS, petE, psbV*, *aroK,* and *psbAI*. These genes and their locations are described in detail elsewhere (Kaneko *et al*., 2001; see also Volume 3 of this series *Genomes and Genomics of Nitrogen-fixing Organisms*). A major striking new result is that the genes are clustered on the chromosome according to their transcription program. The authors call these clusters "expressed islands". They suggest several global mechanisms to account for this pattern, including one that involves the DNA-binding protein HU, which was shown some time ago to disappear from heterocysts (Nagaraja and Haselkorn, 1994). There is much more to do with these arrays and one can hope that the funding agencies will continue to support this work in Japan for some time to come.

7. PROSPECTS

We are, in fact, on the threshold of a new era in heterocyst research, thanks to the determination of the complete genome sequences of both *Anabaena* and *Nostoc*. These genomes reveal dozens of potential regulatory genes, including transcription factors, histidine kinases, response regulators, and protein kinases. There are numerous genes that encode hybrid proteins, which contain domains of both prokaryotic and eukaryotic regulators. Knockouts of these genes will yield strains with useful phenotypes, some of which will be readily manifest, whereas others will be subtle and detectable only by careful studies of transcription. The genome sequences permit the construction of comprehensive microarrays, as we have seen, to follow the transcription program during differentiation of the wild-type strain as well as the mutants made available by knocking out the putative regulatory genes.

The Cu^{2+}-regulated promoter constructs will make it possible to turn any gene on or off. As an example, it is not possible to grow large amounts of mutants in which the heterocyst glycolipids are not made because they cannot fix N_2. But they

could be grown with a Cu^{2+}-dependent wild-type gene complementing the mutation; then, by removing Cu^{2+} from the medium, the wild-type gene could be turned off. This procedure should allow accumulation of the intermediate prior to the blocked enzymatic step, thus, making it available for analysis. The arrays will also make it possible to study gene expression during the establishment of plant/cyanobacterial symbiosis assuming that experimental problems of synchronization can be overcome.

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Chapter 12

CYANOBACTERIAL ASSOCIATIONS

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1. INTRODUCTION

Many cyanobacteria form symbioses with selected prokaryotic and eukaryotic organisms. Such symbiotic partners are found in all kingdoms; they include microorganisms, fungi, algae, plants, and a few smaller animals. For instance, cyanobacteria appear to associate with certain bacteria, with lichenized and non-lichenized fungi, with a range of photosynthetic algae, with lower and higher plants, and even with a few representatives within the animal kingdom, such as sponges, ascidians (either sea squirts or tunicates), and some worms.

Cyanobacteria with symbiotic competence are primarily found in the genus *Nostoc*, which is common in terrestrial environments, although a few other heterocystous cyanobionts (symbiotic cyanobacteria), such as *Calothrix* and *Scytonema* (in lichens and cycads) and *Richelia* (in diatoms), also form symbioses. Non-heterocystous cyanobacteria, such as *Oscillatoria*, *Phormidium,* and unicellular cyanobacteria of the genus *Prochloron*, *Aphanocapsa*, *Synechocystis* are also reported as capable of associating with marine eukaryotes. Therefore, the latter appear to have a wider range of cyanobionts than their terrestrial counterparts. Because marine symbioses are far less studied and far less fully explored, additional "new" cyanobionts and hosts are to be expected from marine studies. There is a great need for more detailed studies of marine cyanobacterial speciation as well as the symbiotic behaviour of the partners.

All hosts that harbour heterocystous cyanobacteria (*e.g.*, *Nostoc*, *Calothrix*, *Scytonema*, and *Richelia*) rely on the N_2-fixation capacity of the cyanobacteria to cover their need for combined nitrogen. There are also reports of N_2 fixation among a few of the non-heterocystous cyanobionts, but this needs to be verified through examination of N_2-fixation activities, a search for the presence of nitrogenase and/or the structural *nif* genes. It is apparent that the driving force for formation of these

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symbioses and the main contribution of the cyanobacterium is fixation of atmospheric N_2 and the provision of fixed nitrogen to the hosts. Here, we will summarise the past and present knowledge on the best characterised N_2-fixing cyanobacterial symbioses (those with fungi, algae and plants) and on their role as symbiotic N_2 fixers. For wider information on cyanobacterial symbioses, the reader is referred to recent reviews (Rai, 1990a; 2002; Osborne, 2002; Meeks and Elhai, 2002).

Table 1. Major Historical Landmarks related to N_2-fixing Cyanobacterial Symbioses

Event	Reference
Cyanobiont:	
Origin of cyanobacteria - about 3.2 Ma ago	see Douglas, 1994
Soil cyanobacteria fix N_2	Frank, 1889; Prantl, 1889
Cultures of *Nostoc* fix N_2	Schlösing and Laurent, 1892
Algae-free *Nostoc* fixes N_2	Kossowitch, 1894; Bouilhac, 1896
Growth of cyanobacteria in N-free media	Beijerinck, 1901
Bacteria-free cultures of cyanobacteria fix N_2	Pringsheim, 1913
Bacteria-free cyanobacteria grow in N-free media	Drews, 1928
Bacteria-free cyanobacteria fix N_2	Fogg, 1942
The genome of *Nostoc* ATCC 29133 sequenced	see Meeks *et al*., 2001
***Symbiosis*:**	
Coralloid roots on cycads	Schacht, 1853
Cyanobacteria in coralloid roots of cycads (*Anabaena*) and in stems of *Gunnera*	Reinke, 1872; 1873
Nostoc in a liverwort	Janczewski in Prantl, 1889; Molisch, 1926; 1940
The *Geosiphon* symbiosis discovered	von Wettstein, 1915;
Cyanobionts fix N_2 when isolated	Winter, 1935
$^{15}N_2$ fixation by cyanobionts isolated from cycads	Watanabe and Kiyohara, 1963
^{15}N transfer from a cyanobiont to the host plant	Bergersen *et al*., 1965 (cycads); Silvester and Smith, 1969 (*Gunnera*)
N_2 fixation by cycads, using C_2H_2	Stewart *et al*., 1968
Diminished GS activity in the cyanobiont and transfer of ammonia from cyanobiont to mycobiont, usaing ^{15}N	Stewart and Rowell, 1977

2. HISTORICAL ASPECTS AND LANDMARKS

The first reports on the existence of cyanobacterial interactions with eukaryotes date back to around the mid-19th century (Table 1), although the question of whether these associations were merely coincidental, parasitic, or truly beneficial was debated for a long time and many conflicting studies were published. DeBary introduced the concept of symbiosis in 1872 ("des Zusammenlebens zwei ungleichnamiger Organismen") and, at about the same time, Reinke (1873) provided evidence for the repeated occurrence and consistency of cyanobacterial-plant associations. However, it took almost 100 years before evidence was provided for the *Nostoc-Gunnera* interaction being truly symbiotic, when the ^{15}N fixed by the cyanobiont was retrieved from the host plant (Silvester and Smith, 1969).

Likewise, the taxonomic affiliation of the cyanobionts in plants has changed over the years, but mostly they have been identified as *Nostoc punctiforme*. Recent phylogenetic studies and sequencing of the entire 16S-rRNA gene clearly indicate that cyanobacteria isolated from various host plants belong to the terrestrial genus *Nostoc* and do not form a separate clade within this genus (U. Rasmussen and M. Svenning, unpublished data). Furthermore, it cannot yet be ruled out that all *Nostoc* species may form a plant or fungal symbiosis if the appropriate host and conditions are present. So far, we have no specific marker-character(s), gene(s), or protein(s) that either point(s) to or prove(s) symbiotic competence.

The major cyanobiont is *Nostoc* and, like all *Nostoc* species, those that form fungal and plant symbioses have a complex life cycle as illustrated in Figure 1. The mature filaments are composed of rounded photosynthetically competent vegetative cells. Some of them turn into nitrogenase-containing heterocysts (typically 5-10 %), when experiencing N-depletion (Meeks and Elhai, 2002). In this way, they become independent of a combined-nitrogen source and may live in nitrogen-poor environments, including those apparently offered *in planta*. Under other harsh external conditions, the vegetative cells may be transformed into unicellular akinetes with thick cell walls and numerous storage structures (Sutherland *et al.*, 1979; Adams and Duggan, 1999). Once conditions are again favourable, the akinetes germinate into vegetative filaments. Akinetes have only rarely been encountered in symbiosis, the exception being in certain *Azolla* spp. (see Lechno-Yossef and Nierzwicki-Bauer, 2002).

A more short-term means of escape is the conversion of the vegetative filaments into motile but short-lived hormogonia. This stage is essential for the establishment of most symbioses because hormogonia constitute the "infection unit". Hormogonia differentiation is, therefore, a pivotal part of symbiotic competence in cyanobacteria. Although necessary, it apparently is not sufficient. Other hormogonia-producing genera, such as *Fischerella* (group V; Rippka *et al.*, 1979), have not been found in any plant symbioses (so far) and are non-competent in reconstitution experiments (Johansson and Bergman, 1994). In some cases, the preceding non-motile primordial stage with terminal heterocysts (Figure 1) may function as the infection units (Kluge *et al*., 2002).

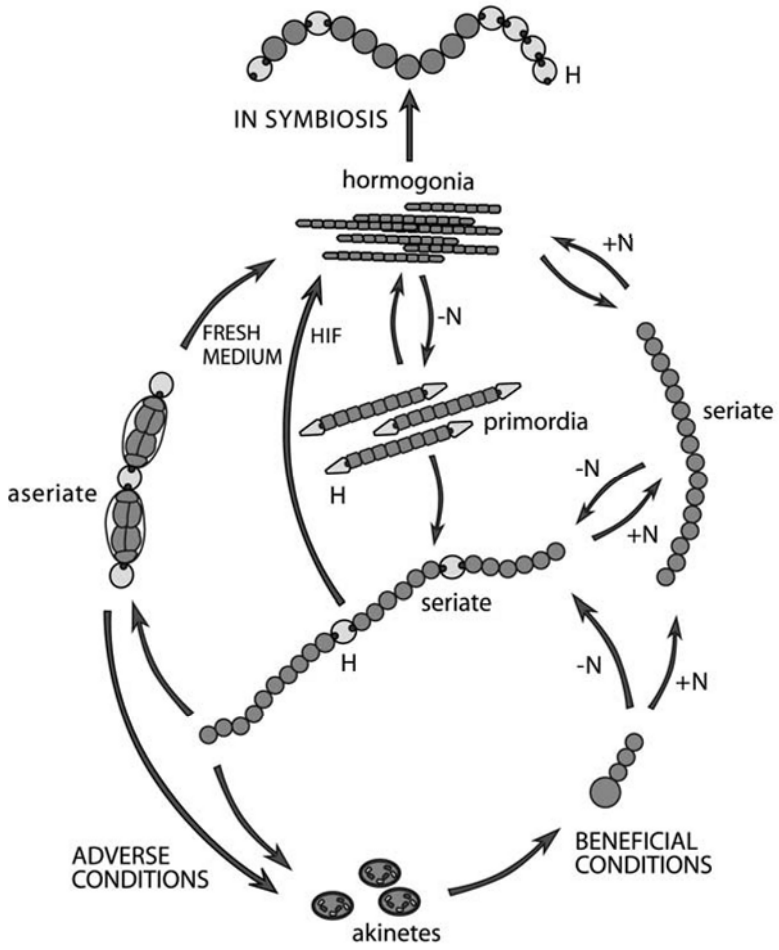

Figure 1. Schematic illustration of the life cycle of Nostoc *when living freely and in fungal/plant symbioses. The vegetative stage is composed of photosynthetically active vegetative cells arranged in filaments. On nitrogen deprivation, thick-walled heterocysts develop and nitrogen fixation commences. Akinetes (spores) are perennial structures with the ability to endure harsh environments, a cell type rarely encountered in symbiosis. Motile small-celled hormogonial filaments develop as a response to changed external conditions (light, dilution, biotic factors-HIFs etc) and are the infection entities in cyanobacterial-plant/fungal symbioses. Also, in the perpetual* Azolla *symbiosis, hormogonia function as inoculum. In the* Nostoc-Geosiphon *symbiosis, the non-motile primordial life stage is the entity `engulfed´ by the fungus. After entering the symbiotic fungal or plant tissue,* Nostoc *filaments develop enhanced frequencies of multiple heterocysts and the cell volume increases. Courtesy of V. Liaimer.*

Symbiotic plant exudates and extracts may both induce and considerably prolong the hormogonial stage beyond their typical 24-48 hours transient life span (A. Liaimer and B. Bergman, unpublished data). Hormogonia-inducing plant factors (HIFs) have been identified, although not chemically characterized, in both extracts of the liverwort *Blasia* (Campbell and Meeks, 1989; see section 5.2) and *Gunnera*-secreted mucilage (Rasmussen *et al*., 1994; see section 8.3.1). Hormogonia that enter the plant, whether they remain extracellular (as in lichens, bryophytes, and cycads) or become intracellular (as in *Geosiphon*, diatoms, and *Gunnera*), are rapidly reconverted into vegetative filaments, although these are now slow-growing and become large-celled (Figure 1). Either some not yet identified host compound or alternatively the conditions offered *in planta* may then elicit the differentiation of multiple heterocysts and convert the cyanobiont from a photoautotrophic self-supportive cyanobacterium into a fixed-N-releasing slow-growing counterpart totally dependent on the host for important nutrients, such as C and Fe (see Bergman *et al*., 1992a; 1992b; Bergman *et al*., 1996; Rai *et al*., 2000).

3. SYMBIOSES WITH DIATOMS (ALGAE)

Diatoms are unicellular algae that are characterized by a silicated cell wall. Some species of diatoms contain either unicellular or heterocystous cyanobacteria as symbionts. Molecular evidence suggests that diatoms arose about 250 Ma ago (Figure 2) and the marine diatoms probably before the freshwater diatoms (Raven, 2002a). However, the time-point when the symbiosis arose is still unknown because of the lack of fossils. On the whole, these diatom symbioses have attracted little attention and our knowledge is scant concerning their occurrence, the infection process, and the metabolic interactions between the partners.

3.1. Unicellular Cyanobionts

The freshwater diatoms *Rhopalodia gibba* and *Epithemia turgida* contain intracellular coccoid cyanobacteria. In *Rhopalodia*, the cyanobiont is located in the cytoplasm but separated by a host membrane (Schenk, 1992). Based on growth in nitrogen-free media and acetylene-reduction assays, the cyanobiont is known to fix N_2 and to increase in cell number when the N/P ratio is lowered (Drum and Pankratz, 1965; Floener and Bothe, 1980; DeYoe *et al*., 1992). Coccoid cyanobacteria have also been reported intracellularly in *Streptotheca indica* and *Neostreptotheca subindica* (see Villareal, 1992).

The symbiosis between the marine diatom *Climacodium frauenfeldianum* and a unicellular cyanobacterium has been described (Carpenter and Janson, 2000). The 16S-rDNA sequence analysis of the cyanobiont indicates a relationship with *Cyanothece* sp. ATCC 51142. Like the *Cyanothece* sp., the cyanobiont may also fix N_2 and benefit the host by the transfer of fixed-N (Carpenter and Janson, 2000; Janson, 2002).

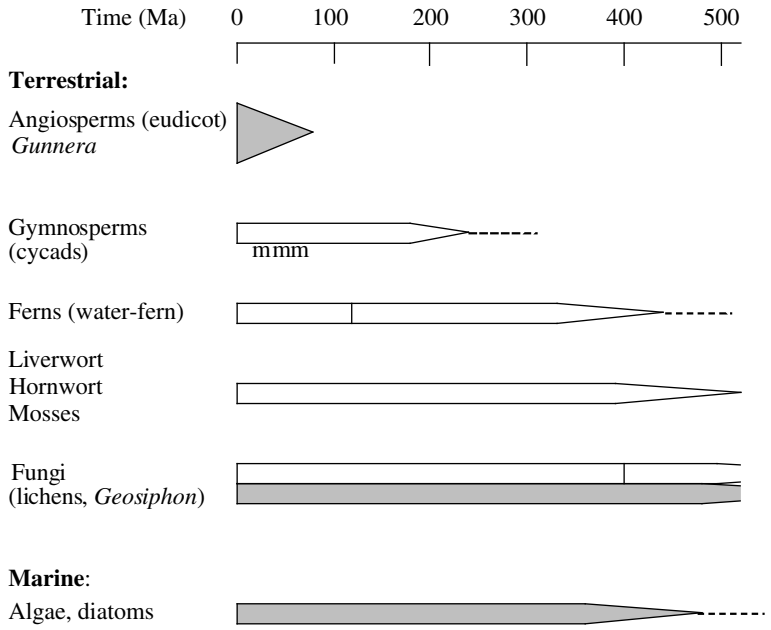

*Figure 2. Organisms acting as hosts for symbiotic cyanobacteria and their proposed time of origin (after Raven, 2002a, b). All known terrestrial and one marine representative are depicted. There seem to be no correlation between ancestry of hosts and the intimacy of the symbiosis. The diatom, certain fungal (*Geosiphon*), and the angiosperm (*Gunnera *spp.) symbioses are intracellular (grey), whereas the cyanobacteria are located extracellularly in the other hosts (white). The angiosperm division is today the largest of the symbiotic plant groups, but the latest to arise. The cycads were much more wide spread 200-150 Ma ago (not shown). The ferns probably arose some 420 Ma ago, while fossils indicate that* Azolla *arose about 120 Ma ago (line). The oldest terrestrial host plants were likely the bryophytes (liverworts, hornworts and mosses). For all the plant symbioses, the time points when the symbioses with cyanobacteria were initiated are unknown. The only fossilised symbiosis discovered today is that of a 400 Ma old cyanolichen (white bar, line) from the Rhynie chert (Taylor* et al.*, 1997), while fungi may have already arisen some 800 Ma ago. The oceans are predicted to harbour numerous symbioses besides the planktonic diatoms shown here (see Carpenter and Foster, 2002).*

3.2. Heterocystous Cyanobionts

The heterocystous cyanobacterium *Richelia intracellularis* (Figure 3a) forms symbioses with the marine diatoms *Rhizosolenia* and *Hemiaulus*. The cyanobiont is

located in the periplasmic space (Villareal, 1992; Janson *et al.*, 1995) and the symbiosis lasts several generations. Although the host and cyanobiont division cycles are not fully synchronized, both partners divide and each daughter diatom cell receives part of the cyanobiont population (Villareal, 1989).

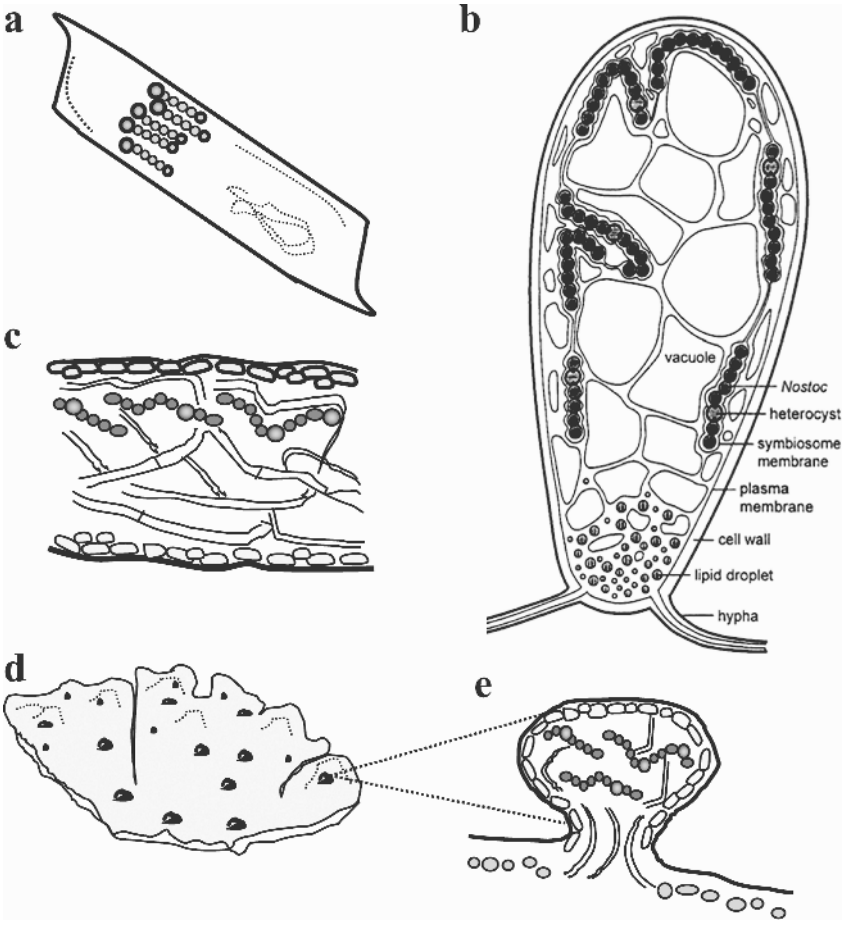

Figure 3. Cyanobacteria in symbioses with algae and fungi.
a) The marine planktonic diatom Rhizosolenia *harbours short filaments of the heterocystous cyanobacterium,* Richelia, *endosymbiotically. b) The fungus* Geosiphon pyriformis *contains* Nostoc *filaments enclosed by the fungal membrane (symbiosome membrane) in the outer region of the fungal bladder. The bladder is formed on engulfment of the cyanobacterium. c) Bipartite lichens contain* Nostoc *in an extracellular layer under the upper fungal cortex (with permission of SpringerVerlag). d) Tripartite lichens may have small wart-like structures scattered on the upper surface known as cephalodia. e) Cephalodia contain* Nostoc *filaments embedded extracellularly among the fungal hyphae, whereas green algae form a photosynthetic layer in the main thallus.*

The cyanobiont fixes N_2 in the symbiosis and the host depends on the fixed-N from the cyanobiont (Mague *et al.*, 1974; Villareal, 1990). Immuno-labelling studies have shown that the nitrogenase is located only in the heterocysts of the cyanobiont and the primary ammonia-assimilating enzyme, glutamine synthetase (GS), is repressed in the cyanobiont (Janson *et al.*, 1995). This situation is consistent with N_2 fixation and release of fixed-N by the cyanobiont because of the insufficient GS to assimilate all the ammonia generated during N_2 fixation. N_2 fixation by this symbiosis is believed to be of great importance in the nitrogen budget of tropical oceans (Carpenter *et al.*, 1999). Blooms covering over 100,000 km^2 of nutrient-poor ocean surfaces have been observed (Carpenter *et al*., 1999). Both the diatom host and the cyanobiont are potentially capable of CO_2 fixation. Early studies indicated that CO_2 fixation in the symbiosis was at the level of the cyanobiont only (Weare *et al.*, 1974). However, there is no evidence that the diatom host depends on the fixed-carbon from the cyanobiont.

4. SYMBIOSES WITH FUNGI

Symbioses between N_2-fixing cyanobacteria and fungi can be grouped into two types: (i) endocytobiotic symbiosis with the *Geosiphon pyriformis* (Kütz) v. Wettstein, a member of Glomales which are closely related to mycorrhizal fungi; and (ii) ecto-symbioses with members of Asco- and Basidiomycetes (Cyanolichens).

4.1. The Nostoc-Geosiphon *Symbiosis*

Kützing discovered the *Nostoc-Geosiphon* symbiosis (Figure 3b) in 1862 and named it *Botrydium pyriforme* (a siphonal alga). Later, von Wettstein (1915) considered it as a symbiosis between *Nostoc* and a heterotrophic siphonal alga. It was not until 1933 that it was correctly identified. Investigations of this symbiosis under controlled conditions became possible only after the organism was cultivated in the laboratory (Mollenhauer and Mollenhauer, 1988).

Reports of the occurrence of *Geosiphon* in nature are rare, with one known site of abundance near Bibergemünd, in the Spessart region of Germany. The dark olive-green bladders comprise a symbiosis between a coenocytic fungus and *Nostoc* (Knapp, 1933). Detailed examination of *Geosiphon*, which appear at the soil surface, is difficult because they are too small and difficult to detect by the naked eye. Hence, the use of molecular probes for its detection in natural habitats should be advantageous. In nature, *Geosiphon* grows in close proximity with mosses and with liverworts/hornworts (*e.g.*, *Blasia* and *Anthoceros*).

It grows in phosphate-poor soils and requires constant humidity but not waterlogging. Excessive fertilisation of the land or high precipitation leads to disappearance of the *Geosiphon* bladders. It has been suggested that a wider interaction may occur among *Geosiphon*, *Nostoc, Anthoceros/Blasia* and the roots of higher plants in the vicinity, forming a large associative consortium (Kluge *et al*., 2002).

4.1.1. The Partners

The cyanobiont in the *Geosiphon* bladders has been identified as *Nostoc punctiforme*. Many but not all strains of *N. punctiforme* form a symbiosis with *Geosiphon*, indicating a certain degree of specificity and recognition between partners. *N. punctiforme* also forms symbioses with liverworts and hornworts (Adams and Duggan, 1999).

The mycobiont is *Geosiphon pyriformis* (Kütz) v. Wettstein, a coenocytic fungus whose mycelium spreads in the uppermost layers of damp, oligotrophic loamy soils. It produces white opaque spores with morphological and ultrastructural features similar to arbuscular mycorrhizal (AM) fungi. Analysis of the SSU rRNA genes of *Geosiphon* has further confirmed its relatedness to AM fungi and together they have been put under the phylum Glomeromycota (Gehrig *et al.*, 1996; Schüßler *et al.*, 2001a; 2001b). Although the cyanobiont has been isolated and cultured outside the mycobiont, it has not yet been possible to culture *Geosiphon* free from *Nostoc*.

Is the *Geosiphon-Nostoc* symbiosis a lichen? Although Knapp (1933) considered it so, Kluge *et al.* (2002) think otherwise because of its apparant differences from lichens, *e.g.*, different mechanisms of vegetative propagation, the aseptate nature of the mycobiont, and the sensitivity to dehydration, plus *Geosiphons*'s closeness to AM fungi.

4.1.2. Developmental Aspects

Geosiphon and *N. punctiforme* occupy similar natural habitats. Upon contact with *Nostoc*, the mycobiont hyphal tip incorporates the cyanobiont by endocytosis and develops into a pear-shaped multinucleate bladder (1-2 mm long and 0.3 mm in diameter). The cyanobiont remains compartmentalised in this bladder (Figure 3b). The bladder has never been observed without the cyanobiont. Initiation of the *Nostoc-Geosiphon* symbiosis and aspects of its development have been studied in detail (Mollenhauer *et al*., 1996; see also Schüßler and Kluge, 2001).

N. punctiforme also undergoes the characteristic life cycle in its free-living state (Figure 1). One of the stages is the immobile form referred to as "primordium". The primordial stage develops from the motile hormogonia. Upon contact with the primordium, portions of *Geosiphon* plasma bulge out from the tip of the hypha to enclose several adjacent cells of the primordium, but always excluding the heterocysts. The hyphal tip swells forming a "bladder" (Figure 3b), which remains coenocytic with the rest of the mycelium. Each such event leads to the formation of a new bladder. The *Nostoc* cells suffer stress, *e.g.*, their photosynthetic pigments bleach, shortly after incorporation into the bladder. However, they fully recover afterwards as manifested by cell multiplication, increased cell size, synthesis of photosynthetic pigments, and development of heterocysts.

Schüßler *et al.* (1997) have studied the recognition process between partners using lectins specific for certain sugars. They synchronised *N. punctiforme* cells by triggering hormogonia formation under red light, followed by a switch to green light illumination that initiated conversion of hormogonia into primordia. Lectin-binding studies showed appearance of mannose in the extracellular slime coincident with the

formation of *Nostoc* primordia. However, the role of a mannose-containing glycoconjugate in the recognition process still remains an open question (see Kluge *et al.*, 2002).

4.1.3. Structural and Metabolic Aspects

Inside the bladder, the *Nostoc* cells reside within a single cup-shaped compartment, the symbiosome, at the periphery of the bladder (Figure 3b). The photosynthetically active *Nostoc* cells occupy the apical part of the bladder. The symbiosome envelope contains chitin and is thought to be of fungal origin. The symbiosome interface between *Nostoc* and the *Geosiphon* cytoplasm resembles the interface found in arbuscular mycorrhiza between the fungus and the plant cell (Schüßler *et al.*, 1996; Schüßler and Kluge, 2001). The *Nostoc* cells in the *Geosiphon* bladder show normal cyanobacterial structures. Bacteria-like organisms (BLO) have been noted in the bladders, hyphae, and spores of *Geosiphon*.

The cyanobiont is photosynthetically active, fixing CO_2 in light (*via* the Calvin Cycle) as well as in darkness (*via* PEP carboxylase) (Kluge *et al.*, 1991). In fact, at certain quantum-flux densities, the *Nostoc* cells in the bladder achieve a much higher quantum yield and faster photosynthetic electron-transfer rates than the cells of the free-living *Nostoc* (Bilger *et al.*, 1994). The cyanobiont forms heterocysts and fixes N_2 (Kluge *et al.*, 1992). The heterocyst frequency however, is similar to that in the free-living *Nostoc*. The BLO of AM fungi have been shown to possess *nif* genes (Minerdi *et al.*, 2001), however, whether BLO associated with *Geosiphon* also possess *nif* genes and fix N_2 needs to be investigated.

Nutrient exchange between the partners in the *Nostoc-Geosiphon* symbiosis has not been investigated apparently due to lack of sufficient cultured material. It has been reasonably assumed that the mycobiont receives fixed-carbon and fixed-nitrogen from the cyanobiont. However, the benefits to the cyanobiont are not so obvious, although it does multiply at a faster rate in the symbiosis indicating plentiful supply of nutrients from the mycobiont. The mycobiont may also maintain homoeostasis of water relations in the vicinity of the cyanobiont (Kluge *et al.*, 2002).

4.2. Cyanolichens

Nearly 12-15% of all known lichen species contain a cyanobacterial partner as the primary or secondary photobiont. Such lichens are known as cyanolichens. Bipartite cyanolichen thalli consist of a fungus (mycobiont) and a cyanobacterial photobiont (cyanobiont) (Figure 3c). The cyanobiont occupies the upper cortical layer in the thallus. Tripartite cyanolichen thalli contain a mycobiont, a green alga as the primary photobiont, and a cyanobiont as the secondary photobiont (Figure 3d-e). In some tripartite cyanolichens, the cyanobiont is located in special structures called cephalodia, which could be either external (on the thallus surface; see Figure 3d-e) or internal (within the thallus). In other tripartite cyanolichens, the cyanobiont occupies a sublayer, quite distinct from the sublayer occupied by the primary photobiont, in the main thallus. Occasionally, bipartite and tripartite

morphologies occur in the same thallus (chimera). Chimeroid lichen thalli with green alga and cyanobacteria as primary photobionts in different parts of the same thallus are called photosymbiodemes, and the corresponding free-living morphologies as chlorosymbiodemes and cyanosymbiodemes, respectively (see Rai, 1990b; Rai *et al.*, 2000; Rikkinen, 2002).

4.2.1. The Partners

Nearly 20% of all known fungi form lichens. Of these, 10% are bipartite and 3-4% tripartite. Most mycobionts are ascomycetes (98%). Rikkinen (2002) provides an exhaustive list. The most common cyanobionts are strains of *Nostoc*. Other heterocystous forms that occur as cyanobionts in cyanolichens include *Stigonema*, *Scytonema*, *Calothrix*, and *Fischerella*. Non-heterocystous cyanobacteria, such as *Gloeocapsa*, *Synechocystis* and *Hyella*, have also been reported as cyanobionts. The green algae, *Trebouxia* and *Coccomyxa*, are the most common primary photobionts in tripartite cyanolichens (see Rai, 1990b; Rikkinen, 2002).

The mycobionts of cyanolichens are fairly selective in their choice of cyanobionts. Only a narrow range of closely related cyanobacterial taxa seem to associate with a specific mycobiont. Among bipartite cyanolichens, only one cyanobacterial strain has been detected in a given thallus. Most tripartite cyanolichens harbor the same *Nostoc* strain in all the cephalodia of a given thallus. However, a notable exception is *Peltigera venosa*, which can house different cyanobionts in different cephalodia (Ott, 1988; Paulsrud and Lindblad, 1998; Paulsrud *et al.*, 1998; 2000). Early reports suggest that some species of *Nephroma* and *Stereocaulon* may also harbor different types of cyanobionts in different cephalodia of a thallus, however, these need confirmation (see Rikkinen, 2002). It has been suggested that the mode of cyanobiont acquisition may play an important role in determining cyanobiont diversity of a lichen thallus (Rai *et al.*, 2000).

Most lichenologists agree that some sort of a signalling and recognition mechanism is involved between the potential partners of the lichen and Rikkinen (2002) has proposed a model. However, the precise details remain illusive although lectins have been suggested to play a role in the recognition (see Rai *et al.*, 2000; Rikkinen, 2002).

4.2.2. The Lichen Thallus

The thalli of most cyanolichens show one of the three growth forms: (i) crustose (undifferentiated crust-like thalli); (ii) foliose (dorsiventral, flat-lobate thalli with a leafy appearance); or (iii) fruticose (shrubby growth forms with round or flat thalli). Primitive lichen thalli are homoiomerous and consist of a loose fungal mycelium enclosing groups of cyanobiont cells. Some resemble a gelatinous cyanobacterial colony penetrated and interwoven by fungal hyphae. Heteromerous thalli are structurally more organised. The cyanobionts are either restricted to a particular layer in the thallus or occur in the cephalodia. In addition to the photobiont layer, which is just below the upper surface of the thallus, there is at least one more defined layer (medulla) that is devoid of photobionts.

The photobiont is extracellular but in close contact with the hyphae (Figure 3c and e). Haustoria are common in some lichens. Immediately below the photobiont layer is the medullar region. The region above the photobiont layer is termed upper cortex and the region below the medulla is the lower cortex. Although homoiomerous thalli are attached to the substratum by the basal hyphae, hyphae originating from the lower cortex form rhizines in heteromerous thalli.

Lichen thalli propagate mainly by vegetative means. Vegetative structures (propagules) develop frequently and help in dispersal and propagation of lichen thalli. Such propagules include phyllidia, isidia, soredia, and hormocystangia (see Nash, 1996; Purvis, 2000; Rikkinen, 2002).

4.2.3. Structural-Functional Modifications of the Cyanobiont

In the lichen thallus, cyanobionts undergo certain structural-functional changes that lead to the establishment of biotrophic nutrient exchange between the partners. The cyanobiont filaments tend to become distorted and sometimes appear to be unicellular. Maximum growth rates occur towards the apical parts of the thallus. Towards the older mature parts of the thallus, cell-division rates decrease and cell sizes increase. Other changes include altered cell shape, fewer polyphosphate granules and corboxysomes, less sheath and thinner cell walls, and altered cell-surface antigens (see Rai *et al.*, 2000). The cyanobionts are photosynthetically active in the lichen thallus. Rates of CO_2 fixation by the cyanobiont increase on going from the younger to the more mature parts of the thallus. However, owing to the limitation of light, CO_2 fixation may be either lacking or very low in cyanobionts of those tripartite lichens in which cephalodia develop either inside (*e.g.*, *Nephroma*) or on the under surface (*e.g.*, *P. venosa*) of the thallus.

There is an overall decrease (>90%) in the glutamine-synthetase (GS) activity of the cyanobiont in lichens. This decrease increases progressively towards the more mature parts of the thallus and the maximum decrease in the GS activity coincides with maximum nitrogenase activity and ammonia release (Rowell *et al.*, 1985; see Rai *et al.*, 2000; 2002). This decrease in activity is due to the repression of GS synthesis in the lichen cyanobionts and the residual GS protein is uniformly distributed in heterocysts and vegetative cells (Janson *et al.*, 1993; see Rai *et al.*, 2000; 2002).

In tripartite lichen thalli, the cyanobionts show an altered spacing pattern and increased frequency of heterocysts. The increase in heterocyst frequency is more towards the central parts of the lichen thallus (Englund, 1977; Rowell *et al.*, 1985; Hill, 1989; Rai *et al.*, 2002). However, in cyanobionts of bipartite lichens, the heterocyst-spacing pattern and frequency remain similar to that in the free-living forms, although an increase from almost zero at the growing lobes is obvious. Heterocystous cyanobionts in both bipartite and tripartite lichens carry out aerobic N_2 fixation in light. The rate of N_2 fixation in tripartite cyanolichens is higher than that in bipartite cyanolichens apparently due to the higher heterocyst frequency. Nitrogenase is located only in the heterocysts of the cyanobionts (Bergman *et al.*, 1986; Janson *et al.*, 1993). Significant levels of N_2 fixation also occur in darkness supported by stored carbon. N_2-fixation rates increase from the younger to the more

mature parts of the thallus and parallel the increase in heterocyst frequency. N_2 fixation declines in very old parts of the thallus (Rai *et al.*, 1983a; 2002; Rowell *et al.*, 1985; Hill, 1989).

The above changes develop in a coordinated manner that optimizes nutrient transfer and maintains a balance between the partners. These changes are the probable result of the special environmental conditions offered by the mycobiont in the lichen thallus. It is unclear whether changes in the cyanobiont are caused directly by the mycobiont.

4.2.4. Nutrient Exchange

Biotrophic nutrient exchange occurs between the lichen partners. Most studies are limited to the transfer of fixed-carbon (from the photobiont to the mycobiont) and fixed-nitrogen (from the cyanobiont to the mycobiont and primary photobiont). In bipartite cyanolichens, the cyanobiont provides both fixed-C and fixed-N to the mycobiont. In tripartite lichens however, the cyanobiont provides only fixed-N and the mycobiont's fixed-C requirement is met by the primary photobiont (green alga).

In bipartite lichens, up to 70% of the fixed-C is released by the cyanobiont as glucose and utilized by the mycobiont. In the mycobiont, the glucose is converted to mannitol, a characteristic of lichenized fungi. This is a mycobiont strategy to sequester carbohydrate and to prevent its use by other partners that are incapable of utilizing mannitol.

In tripartite lichens, there is little or no carbohydrate movement from the cyanobiont to the mycobiont and the cyanobiont is able to meet its own fixed-C requirements. The situation in internal cephalodia and cephalodia occurring on or under the lower surface of the thallus needs investigation. In these situations, the cyanobiont may not be photosynthetically active due to lack of light. For N_2 fixation to occur, fixed-C may have to be made available to the cyanobiont by the primary photobiont (green alga) in the lichen thallus (see Rai *et al.*, 2000; Palmqvist, 2002).

All studies involving cyanolichens with heterocystous cyanobionts have reported N_2 fixation and transfer of fixed-N from cyanobiont to the mycobiont. The cyanobiont releases fixed-N as ammonia, which represents 50-90% of the total N_2 fixed (Rai *et al.*, 1981; 1983b). Repression of GS in heterocysts, the sites of N_2 fixation and primary ammonia assimilation in heterocystous cyanobacteria, is the reason for release of ammonia from N_2 fixation. Ammonia released by the cyanobiont is assimilated by the mycobiont *via* glutamate dehydrogenase (GDH). Very high GDH activity occurs in hyphae close to the cyanobiont cells in either cephalodia (with tripartite lichens) or the main thallus (with bipartite lichens). Along the lichen thallus, rates of N_2 fixation, repression of GS, and ammonia release by the cyanobiont show a correlation and these coincide with elevated levels of GDH in the mycobiont. In tripartite lichens, alanine, which is synthesized in cephalodial mycobionts by assimilation of ammonia received from N_2 fixation by the cyanobiont, moves from cephalodia to the main thallus (see Rowell *et al.*, 1985; Rai *et al.*, 2000; 2002).

5. SYMBIOSIS WITH BRYOPHYTES

Cyanobacteria form either endophytic or epiphytic associations with representatives from the division Bryophyta. This division is divided into three classes: Hepaticae (liverworts), Anthocerotae (hornworts), and Musci (mosses). For recent comprehensive reviews on symbiosis with bryophytes, the reader is referred to Adams (2002) and Solheim and Zielke (2002).

5.1. The Partners

As shown in Figure 2, the division Bryophyta probably represents the first land plants to form cyanobacterial symbiosis (Raven, 2002a; 2002b), although the age of the symbiosis with cyanobacteria is unknown.

5.1.1. Bryophytes

Of the 340 genera of liverworts, only four (*Blasia*, *Cavicularia*, *Marchantia* and *Porella*) form symbiotic associations with cyanobacteria. The genera *Blasia* and *Cavicularia* form endophytic associations with the cyanobacteria being located in a specialised structure, whereas *Marchantia* and *Porella* form epiphytic associations with cyanobacteria. In hornworts, four of the six genera (*Anthocerous*, *Phaeoceros*, *Notothylas*, and *Dendroceros*) form endophytic associations.

The liverworts and hornworts grow as a gametophytic thallus, which is attached to the soil by roots termed rhizoids. In the thallus, the cyanobacterial colonies are visible as blue-green spots of 0.5-1.0 mm diameter (Meeks, 1990). In liverworts (*Blasia* and *Cavicularia*), the cyanobacteria are found in spherical structures, called auricles, on the ventral side of the thallus, whereas in hornworts, the cyanobacteria occupy slime cavities, which are open open to the underside of the thallus (Figure 4a). In all the associations between mosses and cyanobacteria, no special symbiotic structures are formed and the cyanobacteria are considered as epiphytic on the host.

Cyanobacterial-moss interactions are the least studied and also appear to be less intimate associations compared to those between cyanobacteria and hornworts or liverworts. Only on *Sphagnum* mosses have the cyanobacteria been found intracellularly, but then only in dead moss cells. Otherwise, the cyanobacteria display an uneven distribution along the moss plant, with the largest population being recorded in the green upper parts of the thallus. Each new leaf of the moss plant is colonised by hormogonia the year after its formation (Broady, 1979). Thus, it is possible to maintain a cyanobacterial population in the plant regions with the highest light intensity.

5.1.2. The Cyanobiont

The cyanobacteria that form symbioses with liverworts and hornworts belong primarily to the genus *Nostoc*. However, cyanobacteria belonging to the genera *Calothrix* and *Chlorogloeopsis* have shown symbiotic competence with *Phaeoceros* and *Blasia* (West and Adams, 1997). The hornworts *Anthoceros* and *Phaeoceros* and the liverwort *Blasia* can be grown axenically separated from its symbiotic

partner and the symbiosis can easily be reconstituted under laboratory conditions (Rodgers and Stewart, 1977; Enderlin and Meeks, 1983; Meeks, 1988; 1990; Kimura and Nakano, 1990; West and Adams, 1997).

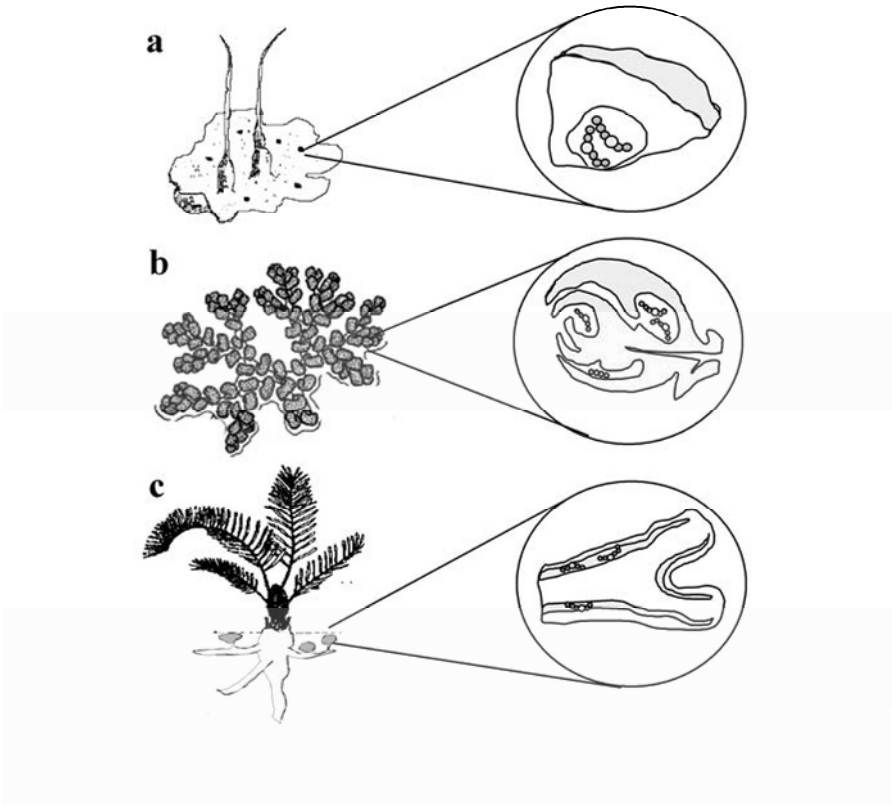

Figure 4. Cyanobacteria in symbiosis with representatives from the divisions Bryophyta, Pteridophyta and Coniferophyta (cycads).
(a). The cyanobacteria are spread in the gamethophytic thallus of the bryophytic hornworts and liverworts and the sites of infection are visualised as small green spots (0.5-1.0 mm diameter). In hornworts (as illustrated), cyanobacteria are located in slime-filled cavities.
(b). In the water fern Azolla, *the cyanobacteria are contained in cavities of the dorsal leaves.*
(c). In cycads, the cyanobacteria occupy special coralloid roots that consist of numerous dichotomously branched structures arising from the lateral roots. The cyanobacteria are restricted to a zone below the outer cortex.
In all the symbioses illustrated, the cyanobacteria are located extracellularly.

Reconstitutions can be made with either the original symbiotic partner or with cyanobacteria originating from another symbiosis, such as from *Gunnera*, cycads, and lichens. Genetic identification of the cyanobacteria from individual cavities in

the thallus of *Phaeoceros*, *Anthoceros,* and *Blasia* have shown that the thallus can be infected by different *Nostoc* strains but only one strain has been found in each individual cavity (West and Adams, 1997; Costa *et al.*, 2001). In associations with mosses, *Nostoc* is dominant but other cyanobacterial genera, such as *Phormidium*, *Microcystis*, *Oscillatoria*, and *Anabaena* have been found (Jordan *et al*., 1978; Solheim and Zielke, 2002).

5.2. Development of the Symbiosis

As with the *Gunnera* and cycad symbioses, in which existing plant structures are infected by the cyanobacteria, the cyanobacteria differentiate into motile hormogonia (Figure 1), before infecting the symbiotic structures in liverworts and hornworts. The hornwort *Anthoceros* has been shown to produce hormogonia-inducing factors (HIF) as a result of fixed-N starvation (Campbell and Meeks, 1989). The identity of the HIF is not known, but its function resembles compounds excreted from *Gunnera*, *Blasia*, and wheat roots (Gantar *et al.,* 1993; Rasmussen *et al.,* 1994; Knight and Adams, 1996; Watts *et al.,* 1999; Watts, 2001). Once hormogonia are induced, they might be guided into the plant-infecting structure by chemoattraction. The liverwort *Blasia* has been shown to excrete signals that not only trigger hormogonia formation but also serve as a chemo-attractant and even increase the speed of hormogonia motility (Knight and Adams, 1996; Watts *et al.,* 1999).

Little is known about the infection process, the chemo-attractants, or the HIFs in mosses. However, the determining factor for deciding between intracellular or epiphytic colonization of *Sphagnum* by cyanobacterium may be dependent on the pH of the environment (Granhall and Selander, 1973). At low pH, cyanobacteria enter into the less acidic environment of the host cells, whereas at higher pH, epiphytic growth predominates.

5.3. Structural and Functional Modifications

After the hormogonia have entered the cavities in the host thalli, different structural and functional modifications, controlled by the host, take place in order to make the symbiotic association stable and functional. First, the hormogonia redifferentiate into vegetative filaments with an elevated heterocyst frequency. In liverworts and hornworts, the heterocyst frequency of the cyanobiont is higher than under free-living conditions (Kimura and Nakano, 1990; Babic, 1996; Bergman *et al*., 1992b). In addition, further differentiation of hormogonia is repressed.

An aqueous extract of *Anthoceros* tissues contains a hormogonia-repressing factor (HRF), which is released into the symbiotic cavity preventing further hormogonia formation. The products of two genes, *hrmA* and *hrmU*, are involved in this process (see Adams, 2002). Mutants, either *hrmA*$^-$ or *hrmU*$^-$, have increased capacity to infect *Anthoceros punctatus*. Further, the flavonoid naringenin, which functions as a signalling molecule in legume-*Rhizobium* communication, induces the expression of *hrmA* in *Nostoc* (Cohen and Yamasaki, 2000). As described

below (section 8.5), *Gunnera* seed rinse may induce *nod*-gene expression in *Rhizobium* (Rasmussen *et al*., 1996).

5.4. Nitrogen Fixation

The metabolic state of *Anthoceros* regulates the size and nitrogen-fixation capacity of the symbiotic cyanobacterium (Enderlin and Meeks, 1983). In addition, the doubling time of the symbiotic cyanobacteria is much slower than its free-living state (Peters and Meeks, 1989; Hill, 1989; Braun-Howland and Nierzwicki-Bauer, 1990). The mechanism of growth control is unknown, but appears to be uncoupled from the fixed-N-limitation signals (Hill, 1989). As in most symbioses, the heterocyst frequency of the cyanobacteria in bryophyte symbioses is enhanced (Adams, 2000). In free-living cyanobacteria, the heterocyst frequency is 5-10%, but when symbiotic, the frequency may reach 30-50% (Rai *et al*., 2000). This phenomenon is observed when the host is photosynthetic, so providing needed energy itself and decreasing the need for cyanobacterial vegetative cells.

The process is presumably controlled by plant signals and is affected by the nitrogen status of the plant (Campbell and Meeks, 1992). The plant might act through inhibition of GS activity, which would result in an inability to assimilate NH_3, fixed-N starvation, and increased heterocyst development even in the presence of a fixed-N source (Stewart and Rowell, 1975). The heterocysts appear to change morphology when in symbiosis with bryophytes, the cause of which is unclear (Meeks, 1990). Changes also occur in the host. In the hornworts *Blasia* and *Anthoceros*, the contact area between the two partners is increased by the growth of filaments from the cavity cell wall of the plant (Rodgers and Stewart, 1977; Duckett *et al.*, 1977; Renzaglia, 1982; Kimura and Nakano, 1990; Gorelova *et al.,* 1996). At least in *Blasia*, these cells display transfer-cell morphology (Duckett *et al.,* 1977).

Another effect of entering into symbiosis is the increased nitrogen-fixation rate that correlates with increased heterocyst frequency. The rate can be several fold higher than that recorded when free-living. The increased nitrogen-fixation rate is supported by photoassimilate obtained from the plant (Steinberg and Meeks, 1991). The fixed-nitrogen is released as NH_3 in both the *Anthoceros* and *Blasia* symbiosis (Rodgers and Stewart, 1974; Stewart and Rodgers, 1977; Meeks *et al.*, 1985). NH_3 uptake by the plant occurs initially through the GS-GOGAT pathway (see Meeks, 1990). The release of NH_3 by the cyanobacteria is a result of decreased activity of GS, presumably as a result either of a post-translational modification or by a decrease in the level of GS in the heterocysts (Joseph and Meeks, 1987; Meeks, 1990). Cyanobacteria retain some of the fixed-nitrogen as can be observed by the presence of the nitrogen-storage bodies; the phycobiliproteins and cyanophycin granules (Rai *et al.,* 1989; Meeks, 1990; Simon, 1987).

In mosses growing in arctic areas, nitrogen-fixation activity is indirectly dependent on the influence of light quality (Zielke *et al.,* 2002). Furthermore, the availability of N, P, Co and Mo, soil moisture and temperature are other factors that affect nitrogen fixation in mosses (Solheim and Zielke, 2002; Solheim *et al.,* 1996; Davey and Marchant, 1983). The rate of CO_2 fixation of the cyanobacteria also decreases when in symbiosis compared to when free-living. Because the level of

Rubisco protein does not change to a similar extent, the activity change may result from post-translational modifications (Steinberg and Meeks, 1989; Meeks, 1990). So, when in symbiosis with bryophytes, cyanobacteria depend on the host for fixed-carbon, probably in the form of sucrose (Steinberg and Meeks, 1991).

6. SYMBIOSIS WITH PTERIDOPHYTES

6.1. The Partners

The water fern *Azolla* is the only known representative within the division Pteridophyta that forms symbiotic associations with cyanobacteria. This symbiosis is unique among the cyanobacterial-plant symbioses becauss it is perpetual, *i.e.*, the cyanobacterium is retained and re-infection of new plants is not needed. It therefore appears that the cyanobacterial-*Azolla* symbiosis is the evolutionarily most advanced, although some other host plants (*e.g.*, Bryophytes; see section 5) arose earlier (Figure 2). This advanced status is indicated by the fact that the cyanobacterium appears incapable of living free of the *Azolla* plant (Lechno-Yossef and Nierzwicki-Bauer, 2002). Compared to the other cyanobacterial-plant symbioses, which are easily separated and reconstituted, the advanced intimacy of the *Azolla*-cyanobacterium interaction may now be at the stage of obligate interdependency (see below).

Yet another peculiarity of the *Azolla* symbiosis, not shared by any of the others, is that bacteria, other than the cyanobacteria, are obligate third partners. The role of these bacteria in the symbiosis is still not understood (Lechno-Yossef and Nierzwicki-Bauer, 2002; see section 6.1.4).

6.1.1. Azolla

The genus *Azolla*, first described by Lamarck in 1783, has traditionally been taxonomically classified depending on spore morphology. However, because not all *Azolla* species form spores and because spore formation can vary with different conditions, this approach has its limitations. Molecular approaches, such as isozymes screening, and RAPD and RFLP analyses, have therefore been introduced (see Watanabe and Van Hove, 1996). The genus *Azolla* is divided into two sections; *Azolla*, often called *Euazolla* (New World species), and *Rhizosperma* (Old World species). The primary basis for this division is the morphology of the spore. Species belonging to the *Azolla* section exhibit three floats on the megaspore apparatus, whereas species in section *Rhizosperma* have nine (Moore, 1969). The seven described species are: *A. caroliniana*, *A. mexicana*, *A. microphylla*, *A. filiculoides* and *A. rubra* (section *Euazolla*), and *A. pinnata* and *A. nilotica* (section *Rhizosperma*).

6.1.2. Reproduction and Continuity of the Symbiosis

Azolla can reproduce either sexually *via* sporocarp formation or asexually through vegetative fragmentation, with vegetative reproduction being the dominant method (Watanabe, 1982; Singh and Singh, 1997; Uheda and Nakamura, 2000).

During sporulation, a male microsporocarp and a female megasporocarp are formed. The megasporocarp contains a distally located colony of cyanobacteria, ensuring that cyanobacteria are transferred to the new generation (see Rai *et al.,* 2000). The packing of cyanobacteria into the sporocarp is facilitated by branched epidermal trichomes, called sporangial pair cells (Perkins and Peters, 1993). Sexual reproduction (sporulation and germination) is dependent on external environmental factors, such as light intensity, temperature, and pH (Kar *et al*., 1999).

When the more common vegetative reproduction takes place, the plant maintains the symbiosis because generative cyanobacterial colonies are present at each branch apex as an inoculum (see Rai *et al.,* 2000). Rapid abscission of branches in response to negative environmental changes enables the plant to spread in a vegetative mode to more beneficial locations (Uheda and Nakamura, 2000).

Sexual hybridisation experiments between different *Azolla* species, as well as transfer of the cyanobionts between different *Azolla* species, have lead to new combinations of *Azolla*-cyanobacteria (Watanabe, 1994; Watanabe and van Hove, 1996). Cyanobacteria from the host *A. microphylla* were, for example, successfully transferred to the new host *A. filiculoides* by exchanging the inducium cap of the megaspore (Lin *et al*., 1989). In addition, sexual hybridizations have been obtained between *A. microphylla* and *A. filiculoides*, (Do *et al*., 1989; Wei *et al*., 1988; Watanabe *et al*., 1993) and between *A. mexicana* and *A. microphylla* (Zimmerman *et al*., 1991).

6.1.3. The Cyanobiont

The cyanobacteria that inhabit the host *Azolla* were identified as the genus *Anabaena* and were named *Anabaena azollae* by Strasburger in 1873. However, it has been proposed that the cyanobiont belongs to the genus *Nostoc* (Meeks *et al*. 1988; Plazinski *et al*. 1990) and the taxonomic affiliation of the cyanobiont is still debated. As mentioned above, all attempts to culture the cyanobacteria from the leaf cavities of *Azolla* have so far failed. Putative cultured isolates always showed both a different morphology and different RFLP patterns (using *glnA, rbcS* and *psbA* as probes) compared to freshly extracted cyanobacteria (Gebhardt and Nierzwicki-Bauer, 1991). However, immobilization of cyanobacteria in synthetic polymers is believed to mimic the conditions within the leaf cavity and might be a valuable technique for isolating and maintaining the cyanobiont (Rajini and Subramanian, 1997).

Nevertheless, in recent years, the cyanobacteria inhabiting the different *Azolla* species have been classified using different molecular markers either directly on isolated DNA from the intact symbiosis or by PCR analysis of intact filaments picked out from the leaf cavities (Plazinski *et al*., 1990; van Coppenolle *et al*., 1993; 1995; Caudales *et al.,* 1995; Kim *et al.,* 1997; Zheng *et al.,* 1999). These investigations show that the cyanobacteria from the *Azolla* and *Rhizosperma* sections are clearly distinguishable.

Moreover, distinctions have also been found between the cyanobacteria infecting the different *Azolla* species within the two sections. These findings, in addition to the continuous maintenance of the symbiosis throughout the life cycle of

the plant, suggest a co-evolution between the cyanobionts and their host plant. However, attempts to detect differences among cyanobacteria within different strains of the same *Azolla* species have so far not been documented. This might be due to either a very high degree of similarity between the cyanobacteria from different strains of the same *Azolla* species or our current methods being inadequate to detect very closely related strains.

6.1.4. Other Endophytic Bacteria

Along with cyanobacteria, other bacteria are also present within the leaf cavity. Using different approaches, such as transmission electron microscopy (TEM) and sequencing of the 16S-rDNA genes, bacteria, such as *Arthrobacter* spp, *Agrobacterium* sp. and *Bradyrhizobium* sp., have been identified (Leonardi *et al*., 1993; Shanno *et al.,* 1993; Serrano *et al*., 1999; Plazinski *et al*., 1990; Lechno-Yossef and Nierzwicki-Bauer, 2002). The bacteria were also observed in the megaspore, indicating that they are transferred together with the cyanobacteria to each new generation and that they are important to the symbiosis (Aulfinger *et al.*, 1991; Carrapico, 1991). However, the function of these bacteria is still unclear. They have been suggested to be responsible for production of the mucilaginous substances present in the leaf cavity (Forni *et al.,* 1992a). They might also influence the plant by production of phytohormones (Forni *et al*., 1992b). Furthermore, some of the bacteria may be involved in nitrogen fixation (Lindblad *et al*., 1991). For a more comprehensive review on these endophytic bacteria, see Lechono-Yossef and Nierzwicki-Bauer (2002).

6.2. The Leaf Cavity

In the *Azolla* plant, the leaves consist of a dorsal and ventral lobe. The ventral lobe is achlorophyllous and floats on the water. The dorsal lobe is chlorophyllous and each contains a comparatively large cavity in which both the cyanobacteria and the bacteria reside (Figure 4b). Approximately 2000-5000 cyanobacterial cells are present within each cavity (Nierzwicki-Bauer *et al.,* 1989). The cyanobacteria are always extracellularly located within the cavity. They are enclosed in mucilaginous material and are present between an inner and outer "envelope" (Uheda and Kitoh, 1991). These envelopes exist even in cyanobacteria-free *Azolla*, indicating that cyanobacteria are not involved in their development. A pore is located in the adaxial epidermis of the cavity and may be involved in gas exchange between the leaf cavity and the atmosphere (Lumpkin and Plucknett, 1982). This pore has a morphology that might prevent particles and organisms from entering the cavity and cyanobacteria from exiting (Veys *et al.,* 2000; 2002).

The growth of the cyanobacteria in the leaf cavities is regulated in a synchronized way so that the cyanobiont never outgrows the host. For instance, when the growth of the host is stopped, cell division of the cyanobacteria is terminated. The growth rate of both partners is highest in the apical parts of the sporophyte and decreases away from the apex. However, the cyanobacterial populations present in the cavities increase from apical regions to older parts and

the cell sizes tend to get larger (Hill, 1977; 1989; Lechno-Yossef and Nierzwicki-Bauer, 2002).

6.2.1. Nitrogen Fixation and Exchange of Nutrients

Azolla has for centuries been used both as feed and as a green manure in China and Vietnam. The first paper on the use of *Azolla* was published in 1725 by Feuillée. The average nitrogen-fixation rate for the *Azolla* symbiosis is 1.0-2.5 kg N/ha/day (Nierzwicki-Bauer, 1990). The nitrogen-fixation rate estimated during the *Azolla*-cyanobacteria symbiosis is 4-18-times higher than with free-living cyanobacteria (Watanabe, 1982). This higher rate correlated to an increase in heterocyst frequency, which can reach as high as 30% in mature cavities during symbiosis (Hill, 1977; Bergman *et al*., 1992b; Rai *et al*., 2000). Heterocyst frequency increases linearly from the young apical leaves towards the basal old leaves where the frequency declines (Hill, 1977), but the number can vary between different *Azolla* species and with diversified growth conditions (Patra and Singh, 1984). The highest nitrogen-fixation rate is found in the middle parts of the plant, corresponding to the high heterocyst frequency observed in this region. Nitrogen fixation in *Azolla* is regulated by light with a lower rate of nitrogen fixation occurring in darkness (Bar and Tel-Or, 1994).

The nitrogen fixed is released as ammonium into the leaf cavity of *Azolla*. Because ammonium levels are low in the leaf cavities in these regions, some efficient removal mechanism must exist, but some of the ammonium is retained in the vegetative cells of the cyanobacteria as cyanophycin granules. A high percent of vegetative cells containing cyanophycin granules have been observed in the middle regions compared to both the apical and basal regions of the plant (Canini *et al*., 1990). The ammonium is most likely taken up by special branched hair cells in the leaf cavity or utilized by other bacteria present in the cavity or both (Canini *et al.,* 1990). The involvement of branched hair cells in nitrogen exchange between the cyanobacteria and its host is documented. In apical leaves, the fixed-nitrogen is found primarily in terminal cells of primary branched hairs (PBH), suggesting that PBH are involved in the transfer of fixed-nitrogen compounds to the apical colony (Albertano *et al.,* 1993). The cyanobacteria excrete *ca*. 50% of their fixed-nitrogen as ammonia, which is assimilated by the GS-GOGAT pathway of the plant and then further metabolised.

Both partners fix CO_2 (Ray *et al.,* 1979), however, sucrose from the plant is transferred to the cyanobacteria and the cyanobacterial rubisco activity is repressed in the middle part of the plant (Rai *et al.,* 2000). Therefore, cyanobacterial CO_2 fixation appears to take place mainly in younger leaves, whereas cyanobacteria in older leaves are dependent on the host for fixed carbon (Rai, 1990a; 1990b).

7. SYMBIOSES WITH CYCADS

The cyanobacterial-cycad symbioses have two unique features. First, they are the only known symbiosis between a gymnosperm and prokaryotic diazotrophs. Second, they are the only symbiosis in which the cyanobacterium invades the roots of a plant. Moreover, all members of this taxon form diazotrophic symbioses.

7.1. The Partners

Cycad symbiosis possibly arose as a single evolutionary event some 250 Ma ago (Raven, 2002a; 2002b), although only fossilised host plants have been encountered so far, not the symbiotic zone with cyanobacteria (see Figure 2).

7.1.1. Cycads

Cycads are restricted to tropical and subtropical parts of the globe, but their distribution was considerably wider 200-150 Ma ago, when the climate was more favourable. Today, their geographical distribution includes most continents, except Europe and Antarctica, but they occur in restricted populations only. For instance, they may be prominent understory plants following fires in Australian eucalyptus forests. Due to their decorative nature, slow growth, and sturdiness, they are often of great interest as ornamental plants in botanical and private gardens.

Cycads belong to the gymnosperms and are therefore not related to palms in spite of their morphological resemblance to that group. Cycads are classified into three families, namely, Cycadaceae, Stangeriaceae and Zamiaceae, and 11 genera, namely, *Cycas*, *Stangeria*, *Bowenia*, *Ceratozamia*, *Chigua*, *Dioon*, *Encephalartos*, *Lepidozamia*, *Macrozamia*, *Microcycas*, and *Zamia*. So far, 160 species have been recognized (see Lindblad and Bergman, 1990; Costa and Lindblad, 2002).

7.1.2 The Cyanobiont

Reinke (Table 1) noticed the association of cyanobacteria with a specific type of cycad roots as early as 1872 and he characterized the cyanobiont as being a "true *Anabaena*". The cyanobacteria that form associations with coralloid roots of cycads have since then been taxonomically ascribed variously to *Anabaena*, *Nostoc punctiforme*, *N. cycadeae*, or simply *Nostoc* sp. (see Grobbelaar *et al.,* 1987; Rasmussen and Nilsson, 2002; Costa and Lindblad, 2002) but, in the majority of cases, as *N. punctiforme*. In fact, the cyanobacterial isolate from *Macrozamia riedli* was selected as the type strain for the genus *Nostoc* under the designation *Nostoc* PCC 73102 (Rippka *et al.,* 1979). Moreover, the first genome of a symbiotically competent cyanobacterium that was sequenced was that of *Nostoc* ATCC 29133, a strain equivalent to *Nostoc* PCC 73102 (Meeks *et al.,* 2001). This will no doubt lead to a much better understanding and, hopefully, definition of the concept cyanobacterial "symbiotic competence".

As in previous morphological studies, recent characterization attempts, using genetic techniques, show that numerous genetically distinct cyanobacterial strains occur in symbiosis with cycads (Costa *et al.,* 1999; Zheng *et al.,* 2002; Rasmussen and Nilsson, 2002). Almost all belong to the genus *Nostoc*, but apparently some occasionally to *Calothrix* also (*e.g.*, as identified in a *Macrozamia* sp.; Grobbelaar *et al.,* 1987). This variation may be due to the fact that cycads are infected *de novo* after seed germination and that the surrounding natural cyanobacterial flora varies geographically and locally and in relation to variations in external conditions. Earlier genetic analyses indicated that only one strain was present in each coralloid root but that different roots of individual plants may harbour different

cyanobacterial strains (see Costa and Lindblad, 2002; Rasmussen and Nilsson, 2002). However, using STRR-PCR DNA fingerprinting, it was recently demonstrated that different cyanobacterial strains might co-exist even within an individual coralloid root (Zheng *et al.,* 2002). However, as other studies have indicated the opposite result, such mixtures of cyanobacteria may either be rare events in cycads or some of the techniques used may not be sensitive enough.

7.2. Development of the Symbiosis

Schacht, as early as 1853, pointed out the occurrence of lateral "coralloid roots" in addition to primary roots on cycads. We now know that these are often, if not always, invaded by cyanobacteria in nature. For instance, Grobbelaar *et al.* (1986) encountered coralloid roots with cyanobacteria in all 33 species of *Encephalartos*, which is indigenous to the Republic of South Africa. The coralloid roots, typically apogeotropic (although this has later been questioned; see Grobbelaar *et al.,* 1986) and adapted for gas exchange, were first characterized as "tubercles" that were formed as a response to the presence of bacteria, other than cyanobacteria (McLuckie, 1922). This idea was later abandoned. Indeed, cyanobacterial infection, and the absence of other bacteria, in the coralloid roots was repeatedly verified (see Schaede, 1944; Wittman *et al.,* 1965; Lindblad *et al*., 1985; Costa and Lindblad, 2002). Coralloid roots are highly modified roots that develop from the pericycle of the primary roots (Bergersen *et al.,* 1965; Ahern and Staff, 1994). On cyanobacterial invasion, the coralloid roots become thicker as the cyanobacteria fill the invaded "zone", which is located below the outer cortex (Figure 4c).

When cross sectioning coralloid roots, a distinct blue-green circular layer, about midway between the stele and the epidermis, can be seen by the naked eye. The former epidermal cells present within the cyanobacterial zone eventually elongate and start to display transfer-cell morphology (Lindblad *et al.,* 1985). A role in exchange of nutrients *via* these cells, extending radially across the cyanobacteria-filled zone, has therefore been inferred. As these host cells only elongate in the presence of invading cyanobacteria, the cyanobacteria may excrete some growth stimulating factor(s) (Wittman *et al.,* 1965; Obukowicz *et al.,* 1981). Interestingly, evidence for the release of the phytohormone auxin (IAA) by symbiotically competent *Nostoc* spp. was provided recently (Sergeeva *et al.,* 2002).

The cyanobacterial zone is, however, discontinuous below the rows of lenticels, which are obvious at the coralloid-root surfaces. Although still unproven, lenticels have been claimed as the entrance for cyanobacterial infection (see Costa and Lindblad, 2002). Nathanielsz and Staff (1975) suggested invasion through cracks in the outer root layers. Still, our knowledge about the mode of infection in cycad roots is scant and the precise stage and mode of entry has been difficult to assess (Ahern and Staff, 1994). One reason may be the comparatively slow germination rates of the often-large cycad seeds, which hinders large-scale infection studies under controlled laboratory conditions. However, a successful isolation of the two symbionts and their subsequent reconstitution has been reported for *Zamia furfuracea* (Ow *et al.,* 1999). Clearly, these aspects need to be studied in greater detail. The development of coralloid roots is genetically determined by the host

plant, being formed in the absence of cyanobacteria, but compatible cyanobacteria may elicit developmental sequences in the symbiotic area of the coralloid roots (Ahern and Staff, 1994). These aspects also need to be further examined.

7.3. Structural and Functional Modifications

As mentioned above, there are some distinct modifications identified in the coralloid roots on entry of the cyanobacterium. This suggests that the cyanobacterium is capable of interfering with the developmental machinery of the plant to initiate the needed symbiotic compartment. Phenolic constituents are present in the mucilaginous material that fills the cyanobacteria-invaded zone, which may also be induced by the presence of the cyanobacterium. These phenolic compounds may function to exclude other microorganisms (Obukowicz *et al.* 1981).

Like all other *Nostoc* spp., the cultured cyanobionts of cycads exhibit the same basic life cycle and cell-differentiation patterns as those illustrated in Figure 1. It is assumed, though not proven, that motile hormogonia are required for cycad-root infections. A most obvious modification in cycad cyanobionts is the enhanced heterocyst frequency. Whether the presumed enhanced *hetR* expression is caused by a high C:N ratio (with carbon provided by the host) or by environmental conditions offered *in planta* (darkness, lower O_2, *etc*.) or by some plant-released compound is still an open question. However, there seems to be a positive correlation between the degree of heterotrophy and the frequency of heterocysts in plant symbioses (Bergman *et al.,* 1992b). A few other modifications of the cyanobiont's cellular structures have been noted (Grilli-Caiola, 1974; 1975; 1980), including rich cyanophycin granules in the cyanobiont of *E. altensteinii* (Grilli-Caiola, 1975), although Neumann (1977) did not find such changes.

7.4. Nitrogen Fixation

Cycads are proposed to provide a valuable source of new fixed-N in the often nitrogen-poor environments they occupy (Halliday and Pate, 1976; Grove *et al.,* 1980). Cyanobacteria-infected coralloid roots incubated under laboratory conditions showed high rates of $^{15}N_2$ fixation in light (5.2 μg N per g root per hour), whereas lower rates were recorded in darkness. The rates are comparable with those of excised soybean-root nodules (Bergersen *et al.,* 1965).

The molar ratio of C_2H_2 to N_2 fixed was estimated to be 5.8:1 for field grown *Macrozamia riedlei* from Western Australia (Halliday and Pate, 1976). Furthermore, a seasonal average in C_2H_2 reduction of 14.8 nmol per g fresh weight per minute was recorded. This rate corresponds to *ca*. 19 kg N per ha per year, a rate that will double coralloid root nitrogen every 8 weeks, but such levels are considerably lower than that of, for example, either cultivated crops or pasture legumes. Still, the levels are considered as remarkable for such a slow-growing system. Activities were higher in winter than in summer and positively correlated with rainfall, whereas soil temperature was less important (Halliday and Pate, 1976). About 1.4-8.4 kg N per ha per year was fixed by *M. riedlei* growing in

eucalyptus forests (Grove *et al.,* 1980). The coralloid roots of all 33 *Encephalartos* species in natural populations in the Republic of South Africa were infected by cyanobacteria and fixed N_2 as shown by both C_2H_2 and $^{15}N_2$ assays (Grobbelaar *et al.,* 1986).

The cyanobionts of cycads are easily isolated from coralloid roots. In fact, the content of the cyanobacteria-filled zone often "pours out" on sectioning the roots. Some days after isolation, when the phycobiliproteins are released from the isolated cyanobionts colouring the media red, they resume growth and soon they function as a free-living *Nostoc*. There are numerous reports on active nitrogen fixation by such isolated and cultured coralloid-root cyanobacteria.

7.4.1. Heterocysts

At the tip of growing coralloid roots, non-heterocystous filaments have been observed, indicating the presence of hormogonia. It has repeatedly been shown that the heterocyst frequencies are enhanced in cyanobionts of cycads (Lindblad *et al.,* 1985; Lindblad and Bergman, 1990; Costa and Lindblad, 2002). The heterocyst frequencies increase from the young towards the basal parts of the coralloid roots and, in the mean time, they undergo some structural changes. Lindblad *et al.* (1985) noted heterocyst frequencies to range from 17-46% along a developmental sequence of *Zamia furfuracea* coralloid root that was growing naturally in Costa Rica. Interestingly, N_2-fixation rates correlated positively only with the occurrence of individually spaced heterocysts and negatively with double or multiple heterocysts, suggesting that the latter are malfunctional. Hence, nitrogenase activities were highest in younger parts along the coralloid roots and negligible in older parts.

7.4.2. Nitrogenase Location

Immuno-histochemistry showed that, although there may be a lower O_2 tension in the cyanobacterial zone (due to high root respiration), nitrogenase is still synthesized only in heterocysts (Bergman *et al.,* 1986).

7.4.3. Nitrogen Assimilation

The nitrogen fixed by cycad cyanobionts is efficiently transferred to the rest of the cycad plant as shown using $^{15}N_2$ (Bergersen *et al.,* 1965). However, unlike some other plant cyanobionts (Rai *et al.,* 2000), cyanobacteria in species of *Cycas*, *Ceratozamia*, and *Zamia* show full GS activity and GS-protein levels (Lindblad and Bergman, 1986). This indicates that the nitrogen fixed by the cyanobiont in cycads, in contrast to other fungal/plant symbioses investigated, is not released to the host plant in the form of NH_3.

To get information about the fixed-N compound released, the cycad xylem sap was collected and analysed for amino acids (Pate *et al.,* 1988). Two distinct patterns were observed. The amino acid glutamine and to a lesser extent glutamate were the principal constituents of the xylem sap in *Bowenia* (Boweniaceae) and *Cycas* (Cycadaceae). In contrast, glutamine and citrulline were the prominent amino acids in xylem sap of *Macrozamia*, *Lepidozamia* and *Encephalartos*, which belong to the family Zamiaceae. Thus, different strategies seem to be used by

different cycad groups. Although the exact N-constituent synthesized and released from the cyanobiont is still to be verified, these differences between host families were further verified by feeding $^{14}CO_2$ to the cultured *Cycas* and *Macrozamia* cyanobionts. Again, the latter synthesized citrulline more readily than the former (Lindblad *et al.,* 1991; Costa and Lindblad, 2002).

7.4.4. Heterotrophy

Ending up in darkness in the coralloid roots of cycads, which occasionally grow down to 50 cm below soil surface, demands a heterotrophic mode of C-assimilation. The cycad plant photosynthesis provides the fixed-carbon as well as all other nutrients required by the cyanobiont. However, like in all other plant symbioses, the plant at the same time needs to restrict growth of the cyanobiont to avoid being "over-grown". Such restriction mechanisms, however, remain unidentified.

8. SYMBIOSIS WITH *GUNNERA*

The *Nostoc-Gunnera* symbiosis differs from the above-mentioned cyanobacterial-plant symbioses in being the only one in which the host plant is an angiosperm. It is also the only one in which the cyanobacterium penetrates plant cells to form an endosymbiosis. These characteristics make the *Gunnera* symbiosis resemble other bacteria-angiosperm symbioses, such as those with either rhizobia or *Frankia*. A marked difference from these angiosperm endosymbiosis is however, that the N_2-fixing microsymbiont in *Gunnera* infects the stems (stolons or rhizomes), not the roots. However, cyanobacteria are capable of infecting roots in gymnosperm host plants (see section 7).

8.1. Angiosperm Endosymbiosis

The reason for the limited occurrence of cyanobacterial symbioses within the large and evolutionarily highly successful angiosperm division is unknown (Figure 2). Symbiotically competent cyanobacteria are not even capable of interacting with the angiosperms (the rosid I clade) that are infected by *Rhizobium* and *Frankia* (Kistner and Parniske, 2002). Likewise, rhizobia and *Frankia* are not found as symbionts in *Gunnera*. These observations suggest that each angiosperm symbiosis has a unique evolutionary background, although they all generate the same final product, an N_2-fixing plant endosymbiosis, and further that each symbiosis must have developed equally efficient, but possibly different, plant-microbe interactive mechanisms.

8.2. Specificity and Diversity

Gunnera is also naturally widespread in wet habitats and found on all continents, except Europe and the Polar regions (Wanntorp *et al*., 2001; Osborne and Sprent, 2002). However, some *Gunnera* spp. has apparently escaped from botanical gardens and is even now invasive in Western Europe (Osborne *et al*., 1991; Osborne and Sprent, 2002). Naturally grown *Gunnera* plants are infected by cyanobacteria

(Figure 5), a phenomenon already observed at the end of the 19th century (Reinke, 1872; 1873; Jönsson, 1894; see Table 1).

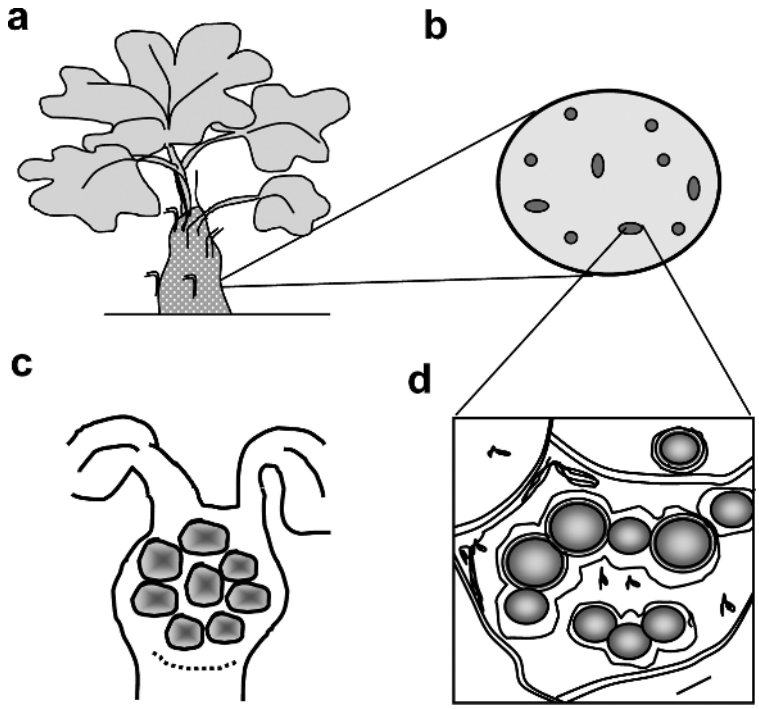

Figure 5. The angiosperm Gunnera *forms N_2-fixing symbioses with* Nostoc *spp. (a). The large* Gunnera *plants may have leaves ranging up to 6 m in diameter. (b). Cross section through the* Gunnera *stem illustrating the numerous* Nostoc*-infection sites (dark spots). (c). Already at the seedling stage, large rounded glands develop, one (or more) at the base of each petiole. The gland is composed of a central and several surrounding papillae. Between the papillae, several mucilage-releasing channels are leading into the interior of the gland. (d). After infection through the channels, competent* Nostoc *filaments are engulfed by* Gunnera *target cells. The* Nostoc *filaments always remain surrounded by the host plasma membrane (dotted line). The intracellular* Nostoc *filaments increase in size and form super-numerous heterocysts (the larger cells).*

Although symbiotic competence may have been more widespread earlier among cyanobacteria, today competence to infect *Gunnera* is, for unknown reasons, restricted to the genus *Nostoc* (Figure 1) (Bergman *et al*., 1992a; Rasmussen and Nilsson, 2002). Although *Nostoc* is common and has a cosmopolitan distribution (Dodds *et al*., 1995), this situation is surprising considering the wide range of extant terrestrial cyanobacteria. It suggests that a horizontal transfer of the genes that

confer symbiotic competence is not frequent either among the cyanobacteria or among plants. This is particularly obvious when one considers the antiquity of the two partners in the *Nostoc*-*Gunnera* symbiosis. The cyanobacteria are estimated to have arisen more than 3 Ba ago (Douglas, 1994) and *Gunnera* is one of the oldest angiosperms known with fossil records dating back some 70-90 Ma (Figure 2), (Raven, 2002a; Osborne and Sprent, 2002; Bergman, 2002). Presumably therefore, the *Gunnera*-*Nostoc* symbioses arose before rhizobial and *Frankia* symbioses (see Kistner and Parniske, 2002).

Although all *Gunnera* isolates are filamentous and heterocystous, the phenotypic range is wide in terms of morphology, pigmentation, colony shape and size. This is particularly apparent when isolates are cultivated on agar plates under laboratory conditions (see Bergman *et al*., 1992a). Since the discovery of this particular symbiosis, the genus *Nostoc* has been the most frequently identified cyanobiont of *Gunnera.* Recent examinations of 16S rRNA also clearly demonstrate that all *Gunnera* isolates examined so far genetically belong to the genus *Nostoc* (Rasmussen and Svenning, 2001). However, PCR-based DNA fingerprinting of a large number of *Gunnera* isolates clearly show that a wide range of *Nostoc* strains are capable of interacting with *Gunnera* species (Nilsson *et al*., 2000), hence explaining the phenotypic plasticity observed earlier (Bergman *et al*., 1992a). Further, a single *Gunnera* plant, and even one gland, may be infected with more than one *Nostoc* strain (Nilsson *et al*., 2000). This demonstrates a high specificity between *Gunnera* and its cyanobionts within the cyanobacterial kingdom because infection is restricted to only one genus (*Nostoc*), but a low specificity between *Gunnera* and the genus *Nostoc* because many strains occur as cyanobionts.

Furthermore, it has been shown, in reconstitution experiments, that *Gunnera* plants may be infected with a variety of cyanobacteria (Bonnett and Silvester, 1981; Johansson and Bergman, 1994). These experiments demonstrated that specificity is not even defined along generic lines because some free-living *Nostoc* spp. were unable to infect. In addition, the closely related genus, *Anabaena*, as well as *Calothrix* and *Fischerella* also failed to infect, although the two latter genera form hormogonia and *Calothrix* spp. may form symbiosis with lichenized fungi and cycads (see Rai *et al.*, 2000). In nature, *Gunnera* is likely to be exposed to a mixture of cyanobacteria, whereas in the reconstitution experiments performed so far, cyanobacteria were only administered one at a time. It would, therefore, be interesting to mix compatible cyanobacterial strains and examine their competitive fitness.

8.3. Development of the Symbiosis

It took almost 100 years before the *Nostoc*-*Gunnera* interaction was shown to be truly symbiotic. This occurred when the $^{15}N_2$ fixed by the cyanobiont was shown to be transferred to the host plant (Table 1; Silvester and Smith, 1969). Since its discovery, the morphological aspects (identified using LM and TEM) of the infection process and the established symbiosis itself have been the major foci of the examination of this symbiosis (see Bergman, 2002). Although we still have only a limited knowledge about molecular mechanisms under-pinning the

establishment of the *Nostoc-Gunnera* symbiosis, several distinct developmental stages from infection to functional maturity of the symbiosis have been identified.

8.3.1. The Mucilage

Initially, the cyanobacterium is attracted to the prominent glands, which develop at each leaf petiole on stems or stolons of *Gunnera* (Figure 5a and 5c). Such stem glands, which readily secrete viscous mucilage, are composed of papillae arranged in a circle with channels penetrating the interior of the stem between the papillae (Johansson and Bergman, 1992; Bergman and Osborne, 2000; Uheda and Silvester, 2001). The mucilage is composed of carbohydrates plus some proteins (Johansson and Bergman, 1992; Rasmussen *et al*., 1994). Fully-grown plants of *G. macrophylla* secrete copious amounts of gland mucilage, often covering the whole scale-bearing apices of the stolons or stems. The mucilage plays a very vital role in the formation of the *Gunnera* symbiosis. The carbohydrates are primarily composed of proteoglucan, arabino-galactan proteins (AGPs; Söderbäck, 1992; Rasmussen *et al*., 1996). It also contains phenolic substances suggested to exclude organisms other than compatible *Nostoc*. However, this function has been questioned because a few other organisms, albeit in low quantities, have been observed in the mucilage as well as in the gland channels using LM and TEM (Schaede, 1951; Towata, 1985; Johansson and Bergman, 1992). Even so, it is clear that the plant knows how to discriminate between compatible and non-compatible cyanobacteria or other bacteria/fungi, because only certain *Nostoc* strains are allowed to enter. The mechanism(s) of discrimination remain unknown.

Nostoc filaments are rapidly converted into non-heterocystous but motile small-celled hormogonia on contact with the mucilage (Figure 1). The hormogonia then glide through the mucilage into the dark interior of the glands (Bergman and Osborne, 2002; Bergman, 2002). Besides inducing hormogonia development, the mucilage also stimulates growth of the cyanobacterium in spite of its low pH (4-5), and induces some new mucilage-specific proteins (Rasmussen *et al.,* 1994). While in the mucilage, the cyanobacterium must experience a chemotactic attraction towards the interior of the gland as it moves away from light. Recognition and acceptance of *Nostoc* by the plant and possibly attachment of *Nostoc* to competent *Gunnera* cells must also take place while the cyanobacterium is in the mucilage-filled channels. To what extent *Nostoc* surface structures and the mucilage *per se* are involved in these events, or whether the mucilage just functions as a carrier of other plant-released compounds, are unresolved questions. Like in bryophyte symbioses (see section 5.2; Campbell and Meeks, 1989), a low molecular weight hormogonia-inducing factor (HIF) (<12 kDa protein) has been identified in the AGP-rich *Gunnera* mucilage (Rasmussen *et al.,* 1994).

Recent studies show that the mucilage not only induces rapid hormogonia differentiation but also prolongs the hormogonial stage (A. Liaimer and B. Bergman, unpublished data). Normally, hormogonia are a short-lived transient stage only, lasting for 20-40 hours under laboratory conditions (Adams and Duggan, 1999; Meeks and Elhai, 2002). In the presence of mucilage, the hormogonial stage may last for weeks, presumably, to enhance the probability of infection. This has

obvious benefit to benefit a plant that is in need of the cyanobacterium as a source of fixed-nitrogen. We are currently searching for the compound that prolongs the hormogonial stage.

8.3.2. Penetration

The plant-cell entry is a unique and important feature of the *Nostoc-Gunnera* symbiosis but little is known about this crucial event. For instance, why does the angiosperm *Gunnera*, as opposed to plant hosts of the other competent plant divisions, allow an intra-cellular penetration by cyanobacteria and how is plant defence reactions avoided? Because the same cyanobacteria that penetrate *Gunnera* cells remain extracellular in cavities of bryophytes (Bonnet and Silvester, 1981; Enderlin and Meeks, 1983; Johansson and Bergman, 1994), the plant apparently dictates the final localization of the cyanobacterium. Whether the plant or the cyanobacterium contributes actively to the penetration is also not clear. The *Gunnera* cells that line the channel start to divide when compatible cyanobacteria are nearby (see Figure 4 in Bergman *et al*., 1996), and it is believed that these are the cells that get infected. This suggests that the cyanobacterium has a role in regulating *Gunnera* division (and thereby the formation of the final symbiotic tissue) and perhaps indirectly plant-cell opening.

Another option is that the phytohormone auxin, which has recently been shown to be released by symbiotically competent cyanobacteria, may have a role (Sergeeva *et al*., 2002). A locally increased IAA level in the *Gunnera* cells along the channels may indeed induce cell division and/or cell-wall "opening". The point of *Nostoc* entry may be at the lysing cell wall between two dividing daughter cells. However, the same IAA-releasing cyanobacteria that infect *Gunnera* do not induce cell-wall openings in host plants other than the angiosperm. Even so, these cyanobacteria stimulate growth of the finger-like hosts cells with transfer-cell morphology found in both cavities of bryophytes (see section 5) and in cycad coralloid roots (see section 7), phenomena that may also be induced by released IAA.

8.3.3. Hormogonia – the Infection Units

Differentiation of hormogonia is crucial for plant infection, but it is not sufficient because, *e.g*., other hormogonia-forming cyanobacteria are not compatible with *Gunnera* (Johansson and Bergman, 1994). Still, it may be that symbiotic competence is "buried" somewhere in the hormogonial stage (Figure 1) of compatible cyanobacteria. As mentioned above, the *Gunnera* mucilage released by the glands is a potent inducer of hormogonia formation (Rasmussen *et al*., 1994). Whether hormogonia capable of responding to plant compounds are genetically different from other hormogonia or whether compatible hormogonia respond differently to plant-released compounds is now under investigation.

The cyanobacterium remains in the motile hormogonial stage until it penetrates the host cells. It is not known whether the hormogonia at this stage may have progressed to the primordial stage (Figure 1), the stage engulfed by the fungus in the *Nostoc-Geosiphon* symbiosis (Kluge *et al*., 2002).

8.4. Plant-Cell Colonization and Adaptations

Functionally, the cyanobacterium is adjusting to the new dark conditions inside the plant stem (Figure 5b) and the *Gunnera* cells. It replaces its photoautotrophic mode of life by a heterotrophic mode and goes from fixing sufficient N_2 to cover its own needs to releasing most of the fixed-N to the plant (see below).

Under laboratory conditions, the infection process (from the external addition of the cyanobacterium to the plantlet to signs of intracellular penetration) lasts for about 2-3 weeks. In spite of penetrating plant cell walls on colonization, the invading filaments are always separated from the cytoplasm by the plant plasmalemma (Figure 5d). Like in rhizobial symbioses, this interface membrane expands massively on *Nostoc* infection. Exchange of metabolites must take place at this interface, with fixed-carbon and other needed nutrients being supplied by the plant to the cyanobacterium and with fixed-nitrogen being transferred from the N_2-fixing cyanobacterium to the plant.

The growing and therefore ever more convoluted and winding *Nostoc* filaments eventually fill up the infected host cells. The *Nostoc* infection is simultaneously spreading to nearby host cells, but the final size of each infection is always adjusted to the size of the host plant (Bergman *et al*., 1992a). Hence, larger plants may have *Nostoc*-infected symbiotic tissues (a mixture of infected and non-infected cells; see Wouters *et al*., 2000) as large as a few cm in diameter (Figure 5c), whereas the infected sites in stolons of *G. magellanica* are very small and restricted to a few mm. The sites of infection are readily seen as dark blue-green spots (Figure 5c) in stems of *Gunnera* as the cyanobiont continues to synthesize its pigments (Söderbäck and Bergman, 1992), which unlike in plants, is a light-independent process in prokaryotes.

Once the *Gunnera* cells are full, growth of the cyanobacterium ceases but their cell volume has increased. This suggests that the plant may prevent cyanobacterial growth (Figure 5d), which may occur *via* either nutritional limitations or by interference with the cell-division machinery but not through cell-volume expansion. Cyanobacteria in *Gunnera* become "entrapped" in the plant cells for prolonged periods of time because the majority of the *Gunnera* species are perennial (Wanntorp *et al.,* 2001). To what extent the cyanobacterium escape on death and decay of the host plant is not known. Perhaps an argument in favour of them regaining free-living competence is the fact that most *Gunnera* cyanobionts are easily isolated and maintained in culture. This also suggests the lack of tight co-evolution between *Nostoc* and *Gunnera* as is apparent in the *Nostoc-Azolla* symbiosis.

8.5. Symbiosis-Specific or -Related Genes

Symbiotically competent cyanobacteria that interact with *Gunnera* are bound to have a symbiosis-related gene pool that is as complex as that of other bacterial endosymbionts. It may even be more complex because the same cyanobacterium may interact with a range of non-angiosperm plant hosts, with all plant organs, and may form both extracellular and intracellular symbioses. Symbiotic gene clusters of

other plant-interacting bacteria are assumed to be spread horizontally and to confer selective advantages. However, the latter may not be the case for *Nostoc* entering *Gunnera* plants, which seem rather to "parasitize" the cyanobacterium. Cyanobacteria in their free-living state are fully self-supportive, able to divide and multiply, able to survive or escape harsh environments (akinetes and hormogonia; see Figure 1), and are capable of fixing all the C and N needed by capturing light energy. In contrast, inside *Gunnera*, they are turned into heterotrophic fixed-N producers with limited possibilities to multiply and escape.

Cyanobacteria may lack the pool of genes required for endosymbiotic interactions with angiosperms other than *Gunnera* because it is not capable of infecting the hosts of either rhizobia or *Frankia*. A search in *Nostoc* for gene homologues to rhizobial *nod* genes showed that the crucial *nodABC* genes were missing, although some other *nod* genes, such as the *nod* box, *nod MN* and *nod EF*, were present (Rasmussen *et al*., 1996). Later however, it turned out that *nodM* in symbiotically competent *Nostoc* merely acts as a housekeeping gene and is not related to symbiosis (Viterbo *et al*., 1999). *Gunnera* extracts do induce *nod*-gene expression in *Rhizobium* (Rasmussen *et al*., 1996) and some *Agrobacterium* genes, such as *chvA*, *chvB* and *picA*, but not *virA* and *virG*, also hybridize with *Nostoc* DNA. This likely reflects the existence of some common chemical-signalling mechanisms, although the significance of these findings needs to be further examined.

More recently, three unknown genes were found to be enhanced in *Nostoc* PCC 9229 when challenged with *Gunnera*-gland mucilage. The genes, termed *hieA*, *hieB* and *hieC* (Liaimer *et al.,* 2001), encode a putative signalling compound (a precursor of a pheromone), an outer-membrane glycoprotein, and a protein that may be involved in adaptation to an acidic environment, respectively. These genes are possibly involved in hormogonia formation and the *hieC*-gene product may help adaptation to the acidic environment offered by the *Gunnera* mucilage. So far, however, no gene(s) specific for symbiosis have been identified in *Nostoc*. Although plasmids can carry "symbiosis gene islands" (*e.g*., the Sym plasmid of rhizobia), the plasmids of symbiotically competent *Nostoc* remain cryptic.

8.6. Nitrogen Fixation

As mentioned above, when *Nostoc* forms a symbiosis with *Gunnera*, it goes through a number of modifications prior to the development of a fully functional and mature symbiosis. Prominent events are related to nitrogen fixation and ammonia assimilation; these include enhanced heterocyst frequencies (Figure 1 and Figure 5d), enhanced N_2-fixation rates, and down-regulation of the GS activity. In parallel, a loss of photosynthetic capacity is apparent and leads to a heterotrophic mode of fixed-carbon acquisition.

8.6.1. Heterocysts and Nitrogenase

During infection, the frequency of heterocysts varies widely from 0% to 80%, depending on the age of the *Gunnera* infection (Figures 1 and 5d). The infectious

hormogonial stage lacks heterocysts, whereas primordia have two terminal heterocysts. After penetration of *Gunnera* cells, intercalary heterocysts appear along the filaments and then rapidly increase to several-fold higher than the number in the free-living stage (Silvester, 1976; Söderbäck *et al*., 1990). In older parts of the symbiosis, the majority of the vegetative cells have in fact become heterocysts.

In free-living cyanobacteria, such as *Anabaena* PCC 7120, *hetR* is the key regulatory gene for heterocyst differentiation (Buikema and Haselkorn, 1991) and *patS* is a signal that, by lateral inhibition, prevents nearby vegetative cells from differentiating into heterocysts (Yoon and Golden, 1998). In this way, a spread of individual heterocysts is formed. Free-living *Nostoc* species display the same phenotype as *Anabaena* with regularly spaced heterocysts. However, in the *Gunnera* symbiosis, the multi-heterocyst phenotype is similar to that seen in either a mutant that over-produces HetR or in a *patS* mutant, both of which develop high frequencies of heterocysts (Figure 1). Also, double to quadruple heterocysts are common in older parts (Bergman *et al*., 1992a). Whether this multi-heterocyst phenotype in symbiosis is induced by a plant compound or by the conditions offered inside the cells of *Gunnera* is not known. The heterocysts in the *Gunnera* symbiosis are the sole sites for nitrogenase as shown by immuno-localization TEM (Söderbäck *et al.,* 1990) and the enzyme is retained even in older symbiotic tissues where N_2-fixation activity is zero.

8.6.2. Nitrogen-Fixation Activities

Cyanobacteria in some New Zealand *Gunnera* species show enhanced N_2-fixation activity compared to when free-living (Silvester and Smith, 1969; Silvester, 1976). The activities however, vary with the age of the symbiotic tissue and distinct profiles occur along, *e.g*., *Gunnera magellanica* stolons (Söderbäck *et al.,* 1990). Activities are low in young newly infected parts, reach a maximum at about 20% heterocysts, and then gradually decline although heterocyst frequencies continue to increase to reach *ca.* 60% further along the stolon. These observations stress the lack of correlation between heterocyst frequency and specific nitrogenase activity after *ca*. 20% heterocysts is reached.

8.6.3. Nitrogen Assimilation

The cyanobacteria are apparently capable of feeding fixed-N to the very large *Gunnera* species *via* N_2 fixation (Bergman *et al.,* 1992a). The nitrogen fixed by free-living cyanobacteria is primarily assimilated by GS. In order to release the nitrogen fixed for the benefit of the plant, this N-assimilation step has been manipulated in all plant symbioses, either at the activity or at the protein level. In the *Nostoc-Gunnera* symbiosis, GS apparently operates and GS activities parallel those of nitrogenase (Söderbäck *et al.,* 1990), *i.e*., they are highest at the growing apex and then decline in older parts. Immuno-TEM showed that the heterocysts contain the same quantity of GS as the vegetative cells. This situation contrasts with the situation in the free-living stage, where the heterocysts have double the amount of GS compared to vegetative cells. Whether this specific reduction in transcription of the *glnA* gene in heterocysts allows enough of the fixed-N to be

transferred to the plant is not known. Most of the fixed-N is released as NH_4^+ plus some asparagine (Silvester *et al*., 1996). Subsequent transfer of the fixed-N to the plant has been verified using $^{15}N_2$ (Silvester and Smith, 1969). Using $^{15}N_2$ and stem-girdling of stolons, the fixed-N is known to be translocated *via* the phloem to the rest of the plant (Stock and Silvester, 1994), in contrast to other angiosperm symbiosis, where the fixed-N is transported in the xylem. These studies also showed that maximum nitrogenase activity occurs in the more mature parts and that part of the fixed-N is translocated to the apical parts (Stock and Silvester, 1994).

8.6.4. Carbon Acquisition

The main energy source used by symbiotic cyanobacteria to fuel N_2 fixation is the fixed-C provided for by the plant *via* photosynthesis. *Gunnera* has photosynthesis typical of C3 plants (Osborne, 1988). It has numerous vascular bundles connecting the leaves with the symbiotic tissues (see Figure 8A in Bergman *et al*., 1992a) and carbohydrates are transported in the phloem. The ability to grow heterotrophically and to fix N_2 in the dark is a typical characteristic of *Nostoc* spp. and is probably a prerequisite for symbiotic competence with *Gunnera* as host.

Söderbäck and Bergman (1993) showed, using ^{14}C, that carbon fixed by *Gunnera* leaves is rapidly transported to the apical parts of the plant and in particular to the symbiotic tissue with the highest nitrogenase activity. Furthermore, fructose and glucose added exogenously stimulated N_2 fixation in excised *Gunnera* infected tissues (Silvester and McNamara, 1976; Man and Silvester, 1994). The same carbohydrates supported N_2 fixation in darkness for weeks when added to cultures of the *Gunnera* isolate *Nostoc* PCC 9229 (Wouters *et al.,* 2000). It is also possible that the recently discovered *ctg* gene (encoding cyclodextrine glycosyltransferase), belonging to the α-amylase gene family, may have a function in heterotrophic N_2 fixation because its expression is maintained in darkness provided fructose is added (Wouters *et al*., 2003). ^{13}C-glucose has also been shown to be taken up and metabolised by symbiotic tissues in *Gunnera* (Black *et al.,* 2002). Furthermore, after 4 weeks on fructose in darkness, double and quadruple heterocysts were apparent and, unlike the situation in the non-fructose-supplemented dark control with no *hetR* expression, *hetR* was now strongly expressed (Wouters *et al.,* 2000). These data suggest that the conditions offered in *Gunnera* may influence cyanobiont gene expression and thereby lead to the morphological and behavioural modifications observed in the cyanobiont engaged in the *Gunnera* symbiosis.

In what way and to what extent the plant supports the cyanobiont with all the other nutrients that are needed by the cyanobiont is unknown. Likewise, it is puzzling how the plant manages simultaneously both to minimize growth of the cyanobiont and to stimulate high N_2-fixation rates.

9. CREATION OF NEW SYMBIOSES AND PROSPECTS

Considerable progress has been made in understanding the establishment and functioning of natural N_2-fixing symbioses involving cyanobacteria. However,

much of the work has focused on the cyanobionts only and very little on the plant hosts. For the creation of N_2-fixing crop plants through either symbiosis or association, it is imperative that this research imbalance is addressed earnestly. Particular emphasis is needed on the *Gunnera-Nostoc* symbiosis because *Gunnera* is the only angiosperm (like most agriculturally and economically important plants) known to enter into symbiosis with N_2-fixing cyanobacteria.

Considering that, during the natural evolution of cyanobacterial-plant symbioses, only a low level of symbiont integration has been achieved, the creation of an obligate and self-perpetuating N_2-fixing symbiosis between cyanobacteria and crop plants will be a difficult proposition. Instead, the creation of facultative associations involving cyanobionts located extracellularly either on or in the plant tissues is a more promising alternative. To do so, first, it has to be borne in mind that modern high-yielding varieties of crops require high input of chemical N-fertilisers over a rather short crop-cycle period (3-4 months). Therefore, it may be feasible to meet only a part of the total fixed-N requirements for the crop. Second, as in natural symbioses, the association has to be established afresh for each generation. This means developing a simple and rapid method for associating the cyanobiont with the crop seed/seedling. Third, diazotrophic cyanobacteria, particularly the symbiotically competent *Nostoc* strains, may be a better choice than *Rhizobium* for creation of N_2-fixing symbioses with cereals. In natural N_2-fixing symbioses, *Nostoc* strains show an ability to colonise a much wider range of plants, plant tissues, and plant organs, and provide N_2-derived fixed-N to the host plants. Furthermore, they are capable of aerobic N_2-fixation, do not require special O_2-protection mechanisms from the plant side, and have flexible modes of C and N nutrition (see Rai *et al.,* 2002).

A number of studies, involving co-culture of *Nostoc*/*Anabaena* with crop seedlings in liquid/sand cultures, have shown that the some *Nostoc* and *Anabaena* strains colonize roots of specific plant cultivars. Although such associations are extracellular and cyanobacteria are mostly located either on the surface or in epidermal cracks, the associated cyanobacteria do seem to support seedling growth in absence of combined nitrogen (see Rai *et al.,* 2000). Studies on colonization of wheat roots by *Nostoc* 2S9B have shown associative N_2 fixation, including substantial N_2 fixation during darkness. Based on an increase in nitrogen content and $\delta^{15}N$ natural-abundance data, it has been suggested that transfer of fixed-N occurs from *Nostoc* to the wheat plant (Gantar *et al.,* 1991; 1995). Treatment with 2,4-D induces root deformation in wheat to provide what are sometimes referred to as "paranodules", which allow enhanced colonization (Gantar and Elhai, 1999). However, the adverse effects of 2,4-D on the plant growth, reproduction and yield are yet to be worked out.

Spiller and co-workers (Spiller and Gunasekaran, 1990; Spiller *et al.,* 1993) showed that fixed-nitrogen-releasing mutants of *Anabaena variabilis* colonize roots and support seedling growth of wheat in co-cultures. This association is extracellular and *A. variabilis* uses fixed-carbon released by the plant while releasing fixed-nitrogen to the plant. Questions, however, remain regarding the relevance of these observations to actual wheat cultivation in the field. For example, wheat is not a transplantation crop and, therefore, cyanobacteria have to

be introduced with the seeds rather than seedlings. Furthermore, it remains to be seen whether successful colonization can be achieved and mutant strains survive in the field.

N_2-fixing cyanobacteria do well in rice fields, colonizing both roots and submerged shoots of rice plants. Rice is a transplantation crop and requires lots of water. A time window occurs between uprooting of seedlings and their transplantation during which cyanobacterial inocula could be associated with the seedlings. Co-cultivation of some soil isolates of *Nostoc* and *Anabaena* with rice seedlings leads to strain- and plant cultivar-specific associations between the cyanobacteria and the rice plants (Svircev *et al.,* 1997). In such associations, cyanobacteria support the growth of rice seedlings in fixed-nitrogen-free media. Successful adsorption and subsequent colonization of rice roots by symbiotic *Nostoc* strains also occurs (see Rai *et al.,* 2000; Nilsson *et al*. 2002). However, these experiments are at an early stage, direct transfer of fixed nitrogen has not been demonstrated, and field experiments are lacking. Much work is needed to enhance symbiotic competence and to introduce improvements, such as enhanced N_2 fixation, nitrogen leakage, herbicide/pesticide resistance, N_2 fixation in presence of combined nitrogen, *etc*., in the cyanobacterial strains that are successful colonizers.

Other aspects in great need of examination are the evolution of cyanobacterial symbioses and the reason for the highly random distribution of cyanobacteria as symbionts in the eukaryotic kingdom. Maybe studies of the latter situation will indicate that these symbioses were once much more common but are now on the retreat. The genetics underlying the phenomenal symbiotic competence held by the genus *Nostoc* spp. is another aspects of great future interest and now that the *Nostoc* ATCC 29133 genome is sequenced (Meeks *et al*., 2001; see also volume 3 *Genomes and Genomics of Nitrogen-fixing Organisms* of this series), as are genomes of several other cyanobacteria, the tools are here.

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Chapter 13

PROSPECTS FOR SIGNIFICANT NITROGEN FIXATION IN GRASSES FROM BACTERIAL ENDOPHYTES

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1. ULTIMATE OBJECTIVE OF NITROGEN-FIXATION RESEARCH – NITROGEN FIXATION IN MAIZE, WHEAT, AND RICE.

Since the discovery of biological nitrogen fixation by Hellreigel and Willfarth (1888), there have been two primary goals of nitrogen-fixation research. The first has been to understand the mechanism of action of nitrogenase as a catalyst for N_2 reduction to ammonia. One long-term objective of this goal is the chemical synthesis of an efficient catalyst for N_2 reduction that could be used for the low-temperature low-pressure synthesis of ammonia. The second goal involves the in-depth understanding of the various associations between nitrogen-fixing bacteria and plants so that (i) the symbiotic association between the legume host and rhizobia can be improved for increased agricultural production and (ii) the delivery of fixed-N to non-legumes can be optimized.

Recent progress in understanding the legume-rhizobia symbiosis has been very impressive and will not be reviewed here. It may be that this knowledge will lead to the induction of nitrogen-fixing root nodules on grasses, such as wheat and rice. However, the discovery of nitrogen fixation in sugarcane over a decade ago has captured the imagination of a growing group of scientists around the world and has led to the study of endophytic nitrogen-fixing bacteria. These are microbes that inhabit the interior of grasses without causing either disease or the induction of any organized symbiotic structures. The progress made to date in the nitrogen-fixation system of sugarcane was reviewed recently (Boddey *et al.,* 2003; see also Chapter 10 of this volume) and will only be briefly summarized here.

Several important questions are now being asked about both the sugarcane system and other endophyte-grass associations that provide high levels of fixed-N to

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important grasses. For example, how can we learn from the sugarcane system? What are the important lessons? Is endophytic colonization regulated in sugarcane? That is, do the diazotrophic endophytes in sugarcane dominate the interior of the plant? If so, how are non-diazotrophic bacteria excluded? Is this an active or passive process? For example, we know that *Gluconacetobacter diazotrophicus*, an important diazotrophic endophyte of sugarcane, is very tolerant to osmotic stress, presumably making it tolerant of high sugar levels in sugarcane tissues. Thus, a passive process can be imagined where those bacteria that can survive the high osmoticum in sugarcane tissues just happen to be able to fix atmospheric N_2. Or it may be that the diazotrophic endophytes of sugarcane produce antimicrobial compounds that exclude other bacteria. A third alternative is that sugar levels in sugarcane are high enough to support non-diazotrophic and diazotrophic bacteria, but not so high as to adversely affect the growth of any of these strains. In this case, the diazotrophs may have an advantage over other strains because they are not limited in fixed-N for growth. All of these situations can be viewed as passive with the host and bacteria not actively participating in forming the association.

However, it may be that this association is actively regulated through one or more signal exchanges between the host and those bacteria that attempt to inhabit the plant interior. For example, the host may be actively excluding the non-beneficial bacteria through host-plant defenses and the diazotrophic bacteria are simply resistant to those defenses. In addition, there may be genetic determinants in the bacteria that are required for host entry. The plant may respond to bacterial signals that induce them to create a better environment for the diazotrophic endophytes.

Unlike the *Rhizobium*-legume symbiosis, where a considerable amount has been learned about the cross-talk between the host and bacterium, very little is known about similar processes in the associations between grasses and diazotrophic endophytes, although this area is beginning to be studied (Vargas *et al.,* 2003). In *Azoarcus* sp. BH72, type IV pili are necessary for surface colonization of rice roots (Dorr *et al.,* 1998), but it is not known whether these pili are essential for the interior colonization of roots. Pectate lyase activity in *Klebsiella* is correlated with the ability to colonize the interior of wheat roots (Kovtunovych *et al.,* 1999). Nevertheless, there is no evidence for any cross-talk between endophytes and their hosts.

Over the last 15 years, most of the research interest on endophytes has concerned their ability to enhance plant growth and/or nutrition. That is, the effect of these bacteria on whole plant growth has been of great interest. However, this has not led to the development of a basic understanding of whether endophytic colonization is regulated. This picture is only now beginning to change. An understanding of the basic biology of endophytic colonization is essential if the ultimate goal of high nitrogen-fixation rates in the world's most important grasses is to be attained based on the sugarcane model.

2. UNDERSTANDING THE BASIC BIOLOGY OF ENDOPHYTIC COLONIZATION: USING *KLEBSIELLA PNEUMONIAE* 342 (Kp342) AS THE MODEL DIAZOTROPHIC ENDOPHYTE.

2.1. Isolation and Identification of Kp342.

In our work, we have focused primarily on a specific model diazotrophic endophyte, *Klebsiella pneumoniae* 342 (Kp342). Diazotrophic *Klebsiella* were first isolated from maize by Palus *et al.* (1996). Strain Kp342 was isolated from a nitrogen-efficient line of maize from the CIMMYT collection. Maize line 342 was cultured in the greenhouse in Madison, WI, and endophytes were isolated as described by Chelius and Triplett (2000b). Kp342 was found to produce the nitrogenase component, NifH, *in planta* when the plants were provided with sucrose. Sucrose is also required for bacterial *nif* expression by diazotrophic endophytes in sugarcane colonized by *Herbaspirillum seropedicae* (James *et al.,* 2002) and in rice plants colonized by *Azoarcus* (Egener *et al.,* 1998; 1999), however, a low level of *nif* expression by *H. seropedicae* LR15 in maize was detected in the absence of any added carbon source (Roncato-Maccari *et al.,* 2003b).

Various physiological tests suggested that Kp342 is a strain of *K. pneumoniae* (Dong *et al.,* 2003a). This was confirmed by DNA:DNA hybridization assays, which showed 78.4% hybridization between DNA from Kp342 and DNA from the type strain of *K. pneumoniae* (Dong *et al.,* 2003a). Values above 70% indicate that two strains are within the same species.

2.2. Fixed-nitrogen Contributions to Wheat

Kp342 can partially relieve nitrogen-deficiency symptoms in greenhouse-grown wheat, whereas the *nif* mutant of Kp342 does not (Riggs *et al.,* 2002). Kp342-inoculated wheat plants were 20% larger and contained 20% more N than either uninoculated plants or plants inoculated with the *nifH* mutant of Kp342. Chlorophyll levels were also much higher in Kp342-inoculated plants. Verification of this fixed-N contribution to wheat by Kp342 is now being sought by ^{15}N isotope dilution analysis.

2.3. Plant-growth Enhancements in the Field

Under standard agricultural conditions, this strain can enhance maize yield but cannot relieve the nitrogen-deficiency symptoms in maize when fixed-N is not provided (Riggs *et al.,* 2001). Kp342 also increases soybean yield in the field (Iniguez and Triplett, unpublished data) and *Arabidopsis thaliana* in the greenhouse (Riggs *et al.,* 2002). These results suggest that Kp342 can enhance plant growth in ways that are independent of nitrogen fixation, however, the mechanism(s) of this growth increase is not known but may involve the production of plant-growth hormones.

2.4. Genome Analysis of Kp342 and Comparisons with a Clinical Klebsiella *Isolate*

Using genomic interspecies microarray hybridization, 3000 genes that are also found in *E. coli* were discovered in this organism (Dong *et al.,* 2001). Ribotyping of Kp342 shows that this strain is within the KpIII group (Dong *et al.,* 2003a). Most clinical *Klebsiella* isolates are within the KpI group. In contrast to the results of Kovtunovych *et al.,* (1999), who found that pectate lyase activity in *Klebsiella oxytoca* is correlated with endophytic entry, Kp342 colonizes the interior of plants despite lacking a pectate lyase gene (Dong *et al.,* 2003a).

Virulence factors for human disease were sought in Kp342. Kp342 lacks type 1 pili, aerobactin production, and a ferric aerobactin receptor (Dong *et al.,* 2003a). All of these are found in clinical isolates of *K. pneumoniae*. In addition, Kp342 only weakly expresses type 3 pili, which are strongly expressed in clinical isolates (Dong *et al.,* 2003a). These data suggest that Kp342 is not an animal pathogen, however, this needs to be tested directly in an animal model.

2.5. Host Specificity, Strain Specificity, and the Number of Bacterial Cells Required for Plant Entry

Kp342 was inoculated onto a variety of host plants at different inoculum levels. Kp342 was found to enter every host tested, including *Medicago truncatula*, *Medicago sativa*, *Arabidopsis thaliana*, *Oryza sativa*, and *Triticum aestivum* (Dong *et al.,* 2003c). These hosts are is in addition to previous work that showed entry into maize (Chelius and Triplett 2000). With each of the five hosts tested by Dong *et al.,* (2003c), a single Kp342 cell was sufficient to result in high interior colonization of the plant just a few days after inoculation. Optimal interior colonization was obtained with 10^3-10^4 cells in the inoculum per plant.

The number of cells per g of root tissue varied with species. With dicots, the number maximized at 10^5 cells per gram, whereas the monocots had considerably higher levels, 10^7-10^8 per g (Dong *et al.,* 2003c). The cause of higher colonization levels in monocots is not known. In each case, controls were included to ensure that the plants were sufficiently surface sterilized prior to the estimation of Kp342 cells in the interior of roots.

The next question of interest was strain specificity for plant entry. That is, does the Kp342 endophyte colonize roots in higher numbers than does either the type strain of *K. pneumoniae* (ATCC13883) or the model strain for *E. coli* genetics, K12? In alfalfa, Kp342 occupied the root interior in numbers two-to-three orders of magnitude higher than those obtained with either K12 or ATCC13883 (Dong *et al.,* 2003b; 2003c). In *Arabidopsis*, Kp342 shows higher interior colonization than ATCC13883 at all but the highest inoculum levels (Dong *et al.,* 2003c). In wheat, colonization numbers are always about 10-fold higher with Kp342 than with ATCC 13883 (Dong *et al.,* 2003c). Only in rice, was there little difference in colonization at all but the lowest inoculum level, where Kp342 colonization was higher (Dong *et al.,* 2003c).

These results demonstrate three important issues in endophytic colonization. First, very few cells are required for plant entry and for high interior-colonization

numbers. Second, a very good colonizer of the plant interior has a very broad host range of entry. This suggests a non-specific mode of entry. And third, closely related strains can differ greatly in their ability to enter the plant interior.

3. ATTRIBUTES NEEDED FOR A MODEL DIAZOTROPHIC ENDOPHYTE.

For the biology of endophytic colonization to advance significantly, model diazotrophic endophytes need to be chosen that can be studied by many investigators from both a multi-disciplinary and a worldwide perspective. To date, there is no consensus on a model endophyte to study. To develop such a consensus, the research community first needs to establish those criteria that are needed for such a model organism. I will propose such criteria and then examine how various strains studied to date fit these criteria.

First, the model diazotrophic endophyte should provide fixed nitrogen to an important plant, preferably a grass species of significant agronomic importance.

Second, the organism should be genetically tractable. The tools commonly used in bacterial genetics should be useful in this strain.

Third, ideally the organism should enhance plant growth in a manner independent of nitrogen fixation. This criterion is important because many endophytes are capable of enhancing crop yield in the presence of fixed-N but the mechanisms for these growth increases are not known.

Fourth, it should have a broad host range so that its interior colonization can be studied in those model-plant species, where mutants and gene-silencing procedures are available.

Fifth, the organism must be recoverable in a culturable state from the host plant at any point in the life cycle of the host. This requirement allows the fulfillment of Koch's postulates as the plant matures and permits accurate quantification of the number of living cells in the plant at any time.

Sixth, the strain should be a poor soil saprophyte and depend on the plant host for survival in the field. This ensures that the effects observed are because of its interior colonization and not simply a result of rhizosphere colonization. It also reduces the ability of the introduced organism to spread in the environment should later work show that it is not the most desirable strain for use in agriculture.

Seventh, the model diazotrophic endophyte should occupy the interior of plants in high numbers at a low inoculum dose, without stressing the host.

Eighth, genetic systems should be available for that plant host which receives fixed-N from the endophyte.

And ninth, the model diazotrophic endophyte cannot be a human, animal, or plant pathogen.

Given these important attributes of a model diazotrophic endophyte, which strains currently studied best fit these criteria?

3.1. Gluconacetobacter diazotrophicus *PAl5*

Perhaps the most important endophyte studied to date is *Gluconacetobacter diazotrophicus* PAl5. First described in 1989 as *Acetobacter diazotrophicus* (Gillis

et al., 1989), this species has been found to inhabit the interior of sugarcane in many countries, including Brazil, Mexico, and India (Gillis *et al.,* 1989; Fuentes Ramirez *et al.,* 1993; Muthukumarasamy *et al.,* 1999).

With respect to the criteria above, PAl5 is known to provide fixed-N to sugarcane (Sevilla *et al.,* 1998; 2001), although it is not certain that this fixed-N comes from bacteria colonizing the rhizosphere and/or the interior of the plant because very little *nif* expression is observed *in planta* (James *et al.,* 2001). Kennedy, Nordlund, and coworkers have shown that PAl5 is very genetically tractable (Sevilla *et al.,* 1997; 1998; Teixeira *et al.,* 1999; Lee *et al.,* 2000; Ureta and Nordlund 2001; 2002; Perlova *et al.,* 2003). Thanks to this work, the *nif* regulon and glutamine metabolism are well understood in this organism. This strain does enhance plant growth independent of nitrogen fixation as shown in both sugarcane and maize (Sevilla *et al.,* 1998; Riggs *et al.,* 2001).

The host range of this species is broad. It is known to inhabit the interior of sweet potato, sweet sorghum, coffee, *Eleusine coracana*, and pineapple (Paula *et al.,* 1991; Jimenez Salgado *et al.,* 1997; Loganathan *et al.,* 1999; Tapia-Hernandez *et al.,* 2000). However, the host range of PAl5 has not been systematically examined. PAl5 can be recovered from the plant in a culturable state and it is also a poor soil saprophyte. It can colonize plants in high numbers but it is not known how many cells are required for plant entry. This last point is not an important issue with sugarcane, which is vegetatively propagated. Furthermore, PAl5 is not a pathogen on any organism studied to date.

Given these characteristics, PAl5 is a very intriguing subject for analysis, but three problems make it less-than-optimal as a model diazotrophic endophyte. First, it is not known to be an endophyte of any major agronomic grass, such as rice, wheat, or maize. Although PAl5 can enhance maize yield in the field (Riggs *et al.,* 2001), it enters maize in very low numbers (Dong and Triplett, unpublished results). Second, it is still not known whether PAl5 fixes N_2 while in the plant. To date, very little *nif* expression has been observed *in planta* (James and Olivares, 1998; Fuentes-Ramírez *et al.,* 1993; James *et al.,* 2001). Third and perhaps the biggest stumbling block to further research with PAl5 is that its primary host of interest is sugarcane, which is not a good model plant. It is difficult to culture aseptically and is difficult to study genetically. Although EST libraries for sugarcane are available, it is very difficult to make crosses because seeds are difficult to obtain and it has an uncertain ploidy level.

3.2. Herbaspirillum seropedicae

Herbaspirillum seropedicae was first described as an endophyte of sugarcane in 1986 (Baldani *et al.,* 1986). In addition to sugarcane, strains of this species are known to inhabit banana, pineapple, and rice (James *et al.,* 2002; Weber *et al.,* 1999). Growth promotion of rice by inoculation with *H. seropedicae* strains is well documented (Baldani *et al.,* 2000; Verma *et al.,* 2001; Gyaneshwar *et al.,* 2002; James *et al.,* 2002; Roncato-Maccari *et al.,* 2003a) as is maize growth enhancement under field conditions (Riggs *et al.,* 2001). These growth effects may be independent of nitrogen fixation (Riggs *et al.,* 2001), but there are clear instances

where nitrogen fixation seems to have benefited plants (Gyaneshwar *et al.,* 2002; James *et al.,* 2002). Moreover, high levels of *nif* expression have been observed in grasses (Roncato-Maccari *et al.,* 2003b).

As mentioned above, the host range of *H. seropedicae* strains is broad. The strains are also easily recovered and culturable from the plant host. There is little evidence that these strains are particularly effective as soil saprophytes. They enter plants in high numbers but the minimum dose needed for high plant colonization is not known. No stress symptoms have been reported upon inoculation. These strains are easily manipulated genetically as shown by the work of Pedrosa and coworkers (Machado *et al.,* 1996; Pedrosa *et al.,* 1997; 2001; Wassem *et al.,* 2002). *H. seropedicae* can induce a hypersensitive response on sugarcane leaves but does not cause disease (Olivares *et al.,* 1997). A close relative, *H. rubrisubalbicans*, does cause disease in sugarcane (Olivares *et al.,* 1997).

All of the above information, however, was gathered from several strains of *H. seropedicae*. At least three strains, LR15, Z67, and Z152, have been important in this work. If any one of these strains could be shown to meet all necessary criteria for a model endophyte, it would be the ideal choice for future work.

3.3. Azoarcus sp. *BH72*

This strain was first described in 1993 as a diazotroph that colonizes the root interior of Kallar grass (Reinhold-Hurek *et al.,* 1993). Kallar grass shows enhanced nitrogen nutrition upon inoculation with BH72 as reflected by reduced natural abundance of ^{15}N with BH72 inoculation compared to inoculation with a *nifK* mutant (Hurek *et al.,* 2002). Isotope-dilution experiments have shown that a small amount of fixed-N can be provided to *Sorghum vulgare* upon BH72 inoculation (Stein *et al.,* 1997). Those plants inoculated with wild-type strain had significantly more N and more biomass than the plants inoculated with the *nifK* mutant. Various genetic analyses of the strain have shown that it is genetically tractable (Egener *et al.,* 2001; 2002; Martin and Reinhold-Hurek, 2002) and it has not been reported as a pathogen on any organism and does not induce stress on plants.

However, the host range of this strain for endophytic colonization is not known beyond rice and Kallar grass. The ability to colonize the interior of sorghum has not been demonstrated. A significant disadvantage in the use of this strain as a model organism is that is appears to enter a non-culturable state a few days after inoculation. Because the cells become unculturable *in planta* (Hurek *et al.,* 2002), it is then difficult to assess with certainty the number of living cells in the host plant over time. Further, the ability of BH72 to survive in soil is not known. It is also not known how many cells are required in the inoculum for plant entry. Moreover, genetics systems are not known for this strain's primary host, Kallar grass, and there is no evidence that the strain can provide fixed-N to any grass but Kallar grass.

3.4. Klebsiella pneumoniae *342*

Kp342 meets all but one of the criteria for a model diazotrophic endophyte. It provides fixed-N to wheat and can increase plant growth in a manner independent

of nitrogen fixation. It is genetically tractable and can infect several hosts with good genetic systems. It is a poor soil saprophyte but is easily isolated and cultured from plant tissues. A single cell in the inoculum can cause very high interior colonization. It is not a pathogen on plants and is not known as a pathogen in either animals or humans. However, it is clearly a strain of *K. pneumoniae*. This species has members that are opportunistic pathogens of humans. Despite the fact that Kp342 lacks at least two virulence factors, animal tests are necessary to ensure that this organism cannot cause disease.

3.5. Which is the Ideal Model Diazotrophic Endophyte?

Given all of the discussion above, no strain studied to date is ideal. However, there are two that meet most of the criteria. If one strain of *H. seropedicae* could be shown to satisfy all of the criteria, it would be the ideal choice for future work. However, since that has not been done, the next best choice is Kp342. The only disadvantage of this strain is that it is closely related to opportunistic pathogens. If Kp342 is not a pathogen on animals, as expected based on its lack of two virulence factors, it would meet all criteria.

The problem with PAl5 is that its best host, sugarcane, is not genetically useful. Working with sterile sugarcane plants is very difficult and it would be very difficult to generate and maintain mutants in sugarcane. In addition, other problems exist with PAl5, such as its inability to express green fluorescent protein.

Azoarcus appears to be the least useful of all strains studied to date, based on: (i) its entry into a non-culturable state once it infects plants; and (ii) its inability to transfer fixed-N to any major agronomic grass species. The inability to culture this strain after invasion makes it very difficult to study the basic biology of the interaction of this strain with plants. It could still be done with tools that can target this organism specifically, but it is much more difficult than just isolating the strain from plants. *Azoarcus* provided no growth benefit to maize under field conditions either with or without added fixed-N (Riggs *et al.,* 2001).

4. FUTURE WORK NEEDED TO REPLACE NITROGEN FERTILIZER WITH DIAZOTROPHIC ENDOPHYTES.

The strain specificity of endophytic colonization as shown by Dong *et al.* (2003b; 2003c) suggests that endophytic colonization is regulated in some manner. This may be by plant-defense mechanisms but there is no published evidence yet in support of this suggestion. Understanding this regulation should allow us to engineer plants and bacteria that provide an optimal number of bacteria within the plant for nitrogen fixation.

The next issue is to ensure that the bacterium can fix N_2 as efficiently as possible *in planta*. There are a variety of means by which nitrogen-fixation rates can be improved in root-nodule bacteria. These have been described by Maier and Triplett (1996). For example, none of the major diazotrophic endophytes studied to date recycle the H_2 produced during the nitrogenase reaction. The presence of a H_2-uptake system in root-nodule bacteria could significantly enhance legume yield (see

references in Maier and Triplett 1996; Iniguez *et al.,* 2004). In addition, optimal expression of *nifA* and *dctABD* in *Sinorhizobium meliloti* enhances alfalfa yield (Bosworth *et al.,* 1994; Scupham *et al.,* 1996) and similar modifications to diazotrophic endophytes may enhance their ability to provide fixed-N to grasses.

A difficult problem to overcome is whether the organism can fix N_2 at the O_2 concentrations present inside the plant. As described above, a few diazotrophs have been shown to express nitrogenase *in planta* but, in nearly every case, an added carbon source, usually sucrose, is necessary for this expression. It may be that sucrose causes increased bacterial respiration, which may drive the local O_2 concentration sufficiently low to permit nitrogenase expression and activity. It is important in future work to determine the *in planta* O_2 concentration and the optimum O_2 concentration for nitrogenase expression by the endophyte. As the O_2 concentration may be too high within the plant to permit a high nitrogenase rate, there may be ways to improve the bacterium's ability to protect nitrogenase under sub-optimal O_2 concentrations. One approach would be to enhance bacterial respiration. In some cases, an endophyte can produce a biofilm on solid medium that serves to protect nitrogenase from O_2 (Dong *et al.,* 2002). It may be possible to induce this biofilm formation *in planta*.

However, increased numbers and increased nitrogen-fixation rates may not be sufficient to fully relieve nitrogen-deficiency symptoms in the plant. The bacterium must secrete a fixed-N source useful to the plant. If that fixed-N source is ammonia, it needs to be assimilated by the plant as rapidly as possible to avoid the toxicity it can cause. If it is another fixed-N source, such as an amino acid, the plant's metabolism must be able to assimilate the amino acid quickly.

Another issue of concern is that non-N_2-fixing endophytes may be competitive with N_2-fixing endophytes for resources within the plant. The diversity of bacteria within a maize root can be very high (Chelius and Triplett 2001), so high in fact that new genera and species of bacteria were discovered there (Chelius and Triplett 2000a; Chelius *et al.,* 2002). It may be necessary to either engineer or discover an endophyte that can exclude non-diazotrophs from entering the plant either through some form of antibiosis or by being better adapted to the resources of the plant interior for which the bacteria are competing.

Each of these problems is testable and each of them can be solved with current technology. The primary hurdle for achieving significant nitrogen-fixation rates in grasses is no longer technical. What is needed is a commitment on the part of a government and/or private firm to provide the resources necessary to solve this problem. Although this problem continues, nitrogen fertilizer continues to be the highest on-farm cost for many cropping systems in the developed world. Nitrogen-fertilizer production continues to consume large quantities of fossil fuels. In many places where N fertilizer is used, runoff of fixed-N is high and causes contamination of drinking water, making it unsafe to drink, particularly for children. In much of the developing world where fertilizers cannot be purchased, yields of many crops remain greatly depressed for lack of adequate fixed-N supplies. If energy costs rise significantly over the next five years, there may be sufficient incentive on the part of society to provide the resources necessary to solve this problem in a concerted effort.

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